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Minireview

MINIREVIEW

Subversion of nutritional immunity by the pathogenic *Neisseriae*

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One sentence summary: The pathogenic *Neisseria* species are obligate human pathogens that have evolved the capability to exploit the human armamentarium dedicated to sequestering transition metals away from bacterial pathogens, an effect known as nutritional immunity.

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ABSTRACT

The pathogenic *Neisseria* species, including *Neisseria meningitidis* and *Neisseria gonorrhoeae*, are obligate human pathogens that cause significant morbidity and mortality. The success of these pathogens, with regard to causing disease in humans, is inextricably linked to their ability to acquire necessary nutrients in the hostile environment of the host. Humans deploy a significant arsenal of weaponry to defend against bacterial pathogens, not least of which are the metal-sequestering proteins that entrap and withhold transition metals, including iron, zinc and manganese, from invaders. This review will discuss the general strategies that bacteria employ to overcome these metal-sequestering attempts by the host, and then will focus on the relatively uncommon 'metal piracy' approaches utilized by the pathogenic *Neisseria* for this purpose. Because acquiring metals from the environment is critical to microbial survival, interfering with this process could impede growth and therefore disease initiation or progression. This review will also discuss how interfering with metal uptake by the pathogenic *Neisseriae* could be deployed in the development of novel or improved preventative or therapeutic measures against these important pathogens.

Keywords: nutritional immunity; *Neisseria gonorrhoeae*; *Neisseria meningitidis*; transition metals; iron and zinc piracy

INTRODUCTION

Two pathogens in the species *Neisseria* exclusively infect humans and cause significant diseases. *Neisseria gonorrhoeae* causes the sexually transmitted infection (STI) gonorrhea and *N. meningitidis* causes meningococcal meningitis and septicemia. Despite extensive nucleotide sequence identity, the two pathogens cause significantly different diseases and differ dramatically in morbidity/mortality and treatment/prevention strategies.

Neisseria gonorrhoeae causes gonorrhea, which can manifest in a multitude of presentations ranging from urethritis in men and cervicitis in women to pelvic inflammatory disease, septic arthritis and even occasionally meningitis (Hook and Handsfield 2008). Auto-inoculation or vertical transmission to a neonate can result in conjunctivitis, which can lead to blindness, if untreated (Kohlhoff and Hammerschlag 2008). Often genital tract infections in women are asymptomatic (Hook and Handsfield 2008), enabling the infection to persist for longer periods of time and to ascend into the upper reproductive tract. Salpingitis in women can lead to permanent fallopian tube scarring and is a significant cause of infertility in sexually active women of child-bearing age (Westrom 1980). Incidence of gonococcal disease is estimated by the CDC to exceed 800,000 cases per year (CDC 2015), making gonorrhea the second most-common reportable infectious disease in the US. Treatment of this STI has been complicated

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by the dramatic rise in antimicrobial drug resistance (CDC 2007, 2011, 2012; Soge *et al*. 2012; Unemo and Nicholas 2012; Hook and van Der Pol 2013; Barbee 2014), facilitated by the fact that *N. gonorrhoeae* is naturally competent and capable of facilely incorporating new genetic resistance determinants into its chromosome under selective pressure. Current recommendations (CDC 2015) for empirically treating uncomplicated gonococcal disease include administration of two drugs: ceftriaxone intramuscularly and azithromycin orally. Gonococcal infections do not result in protective immunity (Hedges *et al*. 1998, 1999), so vulnerable populations are often repeatedly infected, resulting in increased morbidity. Furthermore, no effective vaccine has been developed to prevent gonococcal disease.

By contrast with manifestations caused by *N. gonorrhoeae*, diseases caused by the very closely related pathogen *N. meningitidis* often result in significant morbidity and mortality. *Neisseria meningitidis* disease can manifest as meningitis and/or meningococcemia and can culminate in multisystem organ failure, circulatory collapse and coma within hours of onset of initial symptoms (Thompson *et al*. 2006). Even in survivors of meningococcal disease, the risk of debilitating sequelae, including deafness, blindness and limb amputation, is high (Pace and Pollard 2012). While *N. gonorrhoeae* typically colonizes the genital mucosal epithelium, from which it can ascend into the upper reproductive tract, *N. meningitidis* usually inhabits the nasopharyngeal epithelium, where it can remain and be carried by individuals without causing any disease manifestations (Caugant *et al*. 1994). Crossing the nasopharyngeal epithelium can result in dissemination through the bloodstream, replication of the bacterium and ultimately meningococcemia. Circulating organisms can also permeate the blood–brain barrier, gaining access to the cerebrospinal fluid, where *N. meningitidis* can replicate rapidly. The resulting meningitis can develop rapidly, precipitously leading to coma and death without appropriate therapy (Nadel 2016). The incidence of meningococcal disease has decreased following targeted vaccination campaigns, with recent cases numbering in the hundreds in the last few years, according to CDC reporting data (CDC 2017). Antimicrobial drug resistance has not emerged in *N. meningitidis* as it has with *N. gonorrhoeae*. The mainstay of therapy for meningococcal disease remains penicillin-G, unless the bacterial etiology is unclear, in which case ceftriaxone is the empirical therapy of choice (Nadel 2016). Polysaccharide capsule-based vaccines have been available to protect against meningococcal disease caused by serogoups A, C, W and Y for decades. In the last few years, however, protein-based vaccines were developed to protect against serogroup B strains, which are endemic in industrialized countries. This breakthrough will likely lead to further decreases in incidence and therefore meningococcal morbidity and mortality (Atkinson, Gandhi and Balmer 2016).

While vaccine development efforts proceed towards improving meningococcal vaccines, no gonococcal vaccine has been developed, despite many years of efforts. Similarly, the arsenal of therapies to treat gonococcal disease is dwindling as well. Ideal targets for either vaccines or therapeutic intervention would be conserved in sequence, consistently expressed during infection, required for survival or virulence, and be easily targeted (for example, located on the cell surface). For the *Neisseria* species in particular, the first two criteria, being conserved in sequence and ubiquitously expressed, are difficult to accomplish. The *Neisseriae* are notorious for presenting to the host, and in particular to the host's immune system, surface antigens that are subject to both antigenic and phase variation. The mechanisms of phase and antigenic variation broadly fall into two categories: RecA-dependent and RecA-independent. Pilin variation falls into the former category and is accomplished by non-reciprocal gene exchange from one of many silent loci in the chromosome (*pilS*) into the only expressed locus (*pilE*) (Seifert *et al*. 1994; Serkin and Seifert 1998). Recombination results in a *pilE* gene with a novel DNA sequence, leading to antigenic changes in the protein. Pili are necessary for adherence to mucosal surfaces, natural competence and twitching motility. Many other neisserial antigens are subject to variation by a process known as slipped-strand mispairing (Murphy *et al*. 1989; Connell, Shaffer and Cannon 1990). During DNA replication, short repeat sequences can slip, causing the replicated strand to either increase or decrease in the number of repeats. Often these repeats are located in the coding region for surface antigens. Thus, a change in the number of repeats results in throwing the protein in or out of reading frame. The gonococcus has been referred to as a chameleon (Robinson *et al*. 1989) since virtually all of the surface structures deployed on the outer membrane are subject to one or another form of high-frequency variation.

Attractive targets for vaccine or drug therapy include nutrient transporters (Cornelissen 2008) because they are conserved in sequence, expressed *in vivo*, required for survival and surface exposed. Nutrients acquired by these transporters include the transition metals iron (Fe) and zinc (Zn). Because these metals are necessary for a variety of physiological functions, targeting acquisition of these nutrients is anticipated to be detrimental to the *in vivo* survival of the pathogens. The nutrient acquisition systems deployed by the *Neisseria* species to acquire these necessary metals is the topic of this review.

Nutritional immunity is imposed by mammalian hosts

Living organisms, including bacteria, require transition metals to fulfill a variety of functions. Most bacteria employ Fe as an electron carrier for energy generation and they possess many Fe-S cluster proteins that function in critical redox reactions. Fe is also a cofactor in enzymes involved in oxidative stress tolerance (catalase, for example). Zn is also a cofactor for many enzymes, including oxidoreductases, hydrolases and transferases (Vallee and Falchuk 1993). The concentrations of Fe and Zn are maintained very low in human hosts, with the deployment of an armamentarium of resources (Fig. 1) that function to sequester these metals at such low levels that bacteria succumb to nutrient deprivation. Besides sequestering these metals to suppress microbial growth and replication, host metal-binding proteins (Fig. 1) also serve to maintain the solubility of metals and restrict them to non-reactive states. Iron, in particular, is highly insoluble at neutral pH and has the capacity to form toxic oxygen radicals via Fenton chemistry. The active effort by the human host to restrict the availability of transition metals to invading pathogens has been termed 'nutritional immunity' (Kochan 1973; Weinberg 1977, 1978). This term was initially applied to Fe, but has more recently been expanded to include other transition metals such as Zn and manganese (Mn) (Palmer and Skaar 2016). This review will focus on the sequestration of Fe and Zn, and the neisserial response to this effort, as our current understanding is limited to these two transition metals.

Subsequent to infection by a pathogen, human hosts impose a 'hypoferremic response' in an attempt to sequester Fe (Cassat and Skaar 2013). This process includes extracting Fe out of the plasma, decreasing Fe absorption in the gut, deployment of neutrophils that harbor Fe-sequestering proteins (including lactoferrin) and elicitation of fever that decreases the ability of bacteria to produce Fe-chelating compounds (siderophores, see

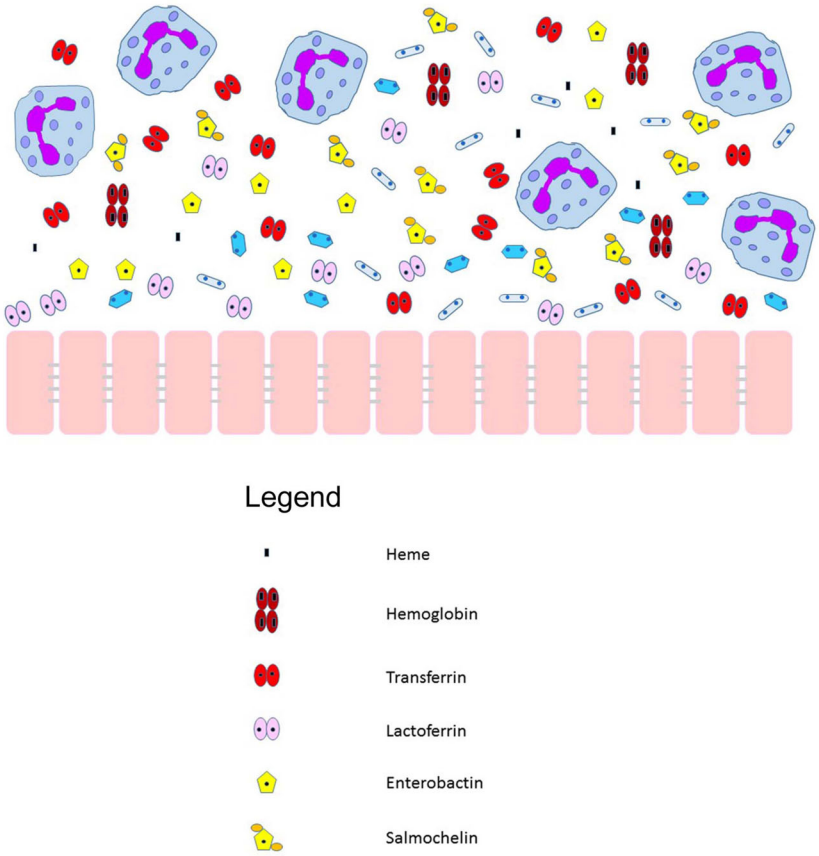

Figure 1. An inflamed mucosa, including neutrophils and antimicrobial substances, encountered by the pathogenic *Neisseriae*. The mucosal membrane, with tight junctions, is shown in pink. Multinucleated cells are meant to represent neutrophils. The key for identification of other metal sequestration substances is shown below the image. Not drawn to scale.

below). This response is coordinated by the hormone peptide, hepdicin. Similarly, 'hypozincemia' is imposed by the human host as a way to diminish free Zn levels that would otherwise be accessible to bacterial pathogens (Liuzzi *et al.* 2005). This response, induced by LPS, is coordinated by the pro-inflammatory cytokine IL-6 and results in Zn sequestration via Zn transporters such as ZIP-14 (Liuzzi *et al.* 2005). Neutrophils, deployed to the site of infection, bring Zn-sequestering S100 proteins, including calprotectin, which makes up ∼40% of the protein content in the neutrophil cytoplasm (Yui, Nakatani and Mikami 2003). Upon degranulation and extrusion of neutrophil extracellular traps (NETs), these Zn-sequestering proteins are in close proximity to bacterial pathogens and function to starve the invader of the nutrients required for growth and survival.

Proteins involved in nutritional immunity include those that sequester both Fe and Zn. Transferrin (Tf) is found at concentrations approximating 30 μM in the serum and is maintained only partially saturated (30%–35%) (Weinberg 1975). The stability constant for Tf binding to Fe is extremely high at 10^{30} (Davis, Saltman and Benson 1962) making bacterial access to the metal bound to this nutritional immunity protein exceedingly difficult. Tf maintains the free Fe levels in serum very low at approximately 10^{-24} M, far below the amount required for bacterial multiplication and survival (approximately 10^{-6} M) (Raymond, Dertz and Kim 2003). Similarly, lactoferrin (Lf) is found in secretions such as tears and milk and in high concentration in neutrophils. Lf is maintained at a lower saturation level, even at low pH, since the primary function of this protein is to absorb any excess Fe and thereby decrease the accessibility to this nutrient by bacterial invaders (Weinberg 2001). It has more recently been appreciated that the S100 proteins calprotectin (complex of A8 and A9), S100B, S100A7 and S100A12 serve a similar sequestering function for Zn (and also Mn and copper [Cu]) (for review, see Zackular, Chazin and Skaar 2015). The S100 proteins are found in high concentrations in neutrophils and on inflamed mucosal membranes (Zackular, Chazin and Skaar 2015).

Cumulatively, the active process of nutritional immunity, the human host's production of metal-sequestering proteins, ordinarily results in concentrations of the transition metals rendered below that needed for bacterial survival. However, the pathogenic *Neisseria* species are masters at overcoming nutritional immunity in that they are capable of using these metal-sequestering proteins directly as metal sources, without the deployment of siderophore intermediates.

Bacteria have evolved diverse approaches for overcoming nutritional immunity

Most bacteria combat Fe-specific nutritional immunity by production and secretion of low molecular weight Fe-chelating compounds called siderophores (Greek for 'iron bearer') (Neilands 1981). Three basic chemistries have been described for Fe coordination: catecholate, hydroxamate and carboxylic acid. Siderophores coordinate Fe with extreme high affinity, sufficient to compete with nutritional immunity proteins such as transferrin for the ferric Fe (Raymond, Dertz and Kim

2003). Both Gram-positive and Gram-negative bacteria secrete siderophores, but the process by which Fe-laden siderophores is internalized by these distinct groups of bacteria depends on the cell envelope structure. Gram-negative bacteria, with the outer membrane barrier beyond the periplasmic space, require an energized system to import Fe-siderophore complexes. The energy to power transport into the cell is provided by the TonB, ExbB and ExbD proteins (Skare *et al.* 1993), which together harness the proton motive force across the cytoplasmic membrane and deliver the energy to the nutrient transporters, situated as β-barrels in the outer membrane (Noinaj *et al.* 2011). These transporters share a common structure comprised of 22 transmembrane β-strands, 11 surface-exposed loops and an amino-terminal plug domain that folds up into the lumen of the β-barrel (Noinaj *et al.* 2011). The extreme amino-terminus of the plug domain represents a point of physical contact between the transporter and TonB, which in some way dislodges or extracts the plug to accomplish transport across the outer membrane (Shultis *et al.* 2006; Gumbart, Wiener and Tajkhorshid 2007). Fe-siderophore transporters are approximately 80–85 kDa in size and share significant sequence identity, particularly in the areas of transmembrane strands and the plug domain (Nau and Konisky 1989), which enables identification of genes that encode these transporters within genome sequence data.

Subsequent to transport across the outer membrane, a periplasmic binding protein (PBP) and ATP-binding cassette (ABC) transport system enables Fe assimilation through the periplasm, across the cytoplasmic membrane and into the cytoplasm. Similar PBP-dependent ABC transport systems are employed by Gram-positive bacteria for import of ferric siderophores into the cell (Sheldon and Heinrichs 2015).

The deployment and subsequent uptake of Fe-siderophores enables bacteria to overcome Fe-specific nutritional immunity. In addition, many bacteria are capable of internalizing siderophores for which they are not producers (for example, see Cornelis 2010). So called xenosiderophore uptake is common and presumably confers an advantage in mixed bacterial populations. Interference with Fe uptake by decreasing siderophore production, which occurs with increased temperature and the host's febrile response, or by sequestering the Fe-siderophore, would advantage the host and 'intensify the iron famine of invaders' (Weinberg 1975). In fact, counter measures are elicited by human hosts to incapacitate Fe-siderophores so that they cannot deliver their payload to bacterial pathogens. Anti-enterobactin antibodies are elicited during infection and anticipated to play a role in withholding siderophore-bound iron (Moore and Earhart 1981). In addition, Lipocalin 2 (also known as neutrophil gelatinase-associated lipocalin or NGAL) is a host protein, present in inflamed sites and in neutrophils, that accomplishes this feat (Flo *et al.* 2004). Lipocalin binds to some Fe-siderophores, for example enterobactin, and prevents pathogens such as *Escherichia coli* from employing this siderophore as an Fe source (Raymond, Dertz and Kim 2003). But in the continuing arms race between invaders and their host, efficient bacterial pathogens have developed an advanced strategy to defeat Fe-siderophore sequestration by lipocalin 2. *Salmonella* Enterica produces not only enterobactin but also a glucosylated version of enterobactin called salmochelin (Muller, Valdebenito and Hantke 2009). The latter siderophore evades sequestration by lipocalin 2 due to the bulky sugar additions and thereby thwarts the host's siderophore scavenging mechanism. These so-called 'stealth siderophores' enable producers to efficiently evade Fe-specific nutritional immunity in the face of lipocalin 2 production.

Similar to the Fe situation, Zn and other metals can be chelated by siderophores as well. The best characterized example of a human pathogen producing a 'zincophore' is *Yersinia pestis*. While yersiniabactin is also an Fe-binding siderophore, recently this multifunctional siderophore has also been shown to bind to and enable internalization of Zn (Bobrov *et al.* 2014, 2017). Siderophores can also complex with other metals including Mn, Cu and Co. Again, analogous to the Fe-uptake systems described above, many bacteria produce a PBP-dependent, ABC transport system, encoded by the *znuABC* genes, which enables highly efficient internalization of Zn and Zn-laden siderophores (Bobrov *et al.* 2014, 2017; D'Orazio *et al.* 2015). The *Neisseria* species are again somewhat unique in that two outer membrane transporters have also been shown to facilitate Zn passage across the outer membrane (see below). Other characterized Zn-uptake systems primarily rely on the high-affinity Zn-uptake system comprised of ZnuABC, with Zn diffusing through the outer membrane via porins. The *Neisseria* species produce a cytoplasmic membrane permease system homologous to ZnuABC (also called MntABC in the literature) (Lim *et al.* 2008), but they furthermore produce cognate outer membrane transporters to accomplish the first step in dedicated Zn transport across the outer membrane.

The *Neisseria* species do not produce siderophores but instead hijack those secreted by other bacteria (xenosiderophores)

The pathogenic *Neisseria* species do not possess the genes that would enable them to produce and secrete siderophores for metal acquisition. However, they do encode a transport system that accomplishes uptake of catecholate-type siderophores including enterobactin, linearized salmochelin (S2) and dihydroxybenzoylserine acid (DHBS) (Hollander *et al.* 2011). The crystal structure of meningococcal FetA suggests that the transporter actually binds to and perhaps directly internalizes ferric Fe (Saleem *et al.* 2012), consistent with a broad specificity for different Fe-containing siderophores (Hollander *et al.* 2011).

The *fet* operon (Carson *et al.* 1999) encodes the components of the siderophore uptake system, including the outer membrane transporter (FetA), a PBP (FetB) and an ABC transport system (FetCDEF) (Table 1; Fig. 2). These genes are co-transcribed in a poly-cistronic operon that is differentially regulated by two different trans-acting factors. The *fet* operon is iron repressed (as are most iron uptake genes) by the repressor Fur. The operon is further positively regulated by an AraC-like regulator, MpeR (Hollander *et al.* 2011). MpeR is iron repressed by virtue of the fact that Fur also represses *mpeR* expression in an Fe-dependent manner (Jackson *et al.* 2010; Hollander *et al.* 2011). Thus, optimal *fet* operon expression includes de-repression by low iron conditions and induction by MpeR activation. None of the recognized siderophore ligands however appear to serve as co-inducing signals with MpeR (Hollander *et al.* 2011).

The *fetA* gene is subject to slipped-strand mispairing and thus exhibits phase variation (Carson *et al.* 2000). Unlike the situation described above in which repeat sequences are located in the coding region, in the case of *fetA*, the repeated C residues are located in the defined promoter region, namely between the -35 and -10 consensus sequences. A change in the number of C residues in this area, due to strand slippage during DNA replication, results in a promoter that expresses either high or low levels of FetA, depending upon the number of nucleotides between the −35 and −10 sequences. Seventeen or 18 nucleotides

Table 1. Metal transport systems involved in overcoming nutritional immunity.

Name in Ngo[a] (alternative name)	Metal/ligand or function	Locus tag in NGO FA1090	Homolog in Nme[b] MC58 (alternative name)	*Neisseria* locus tag	MW[c]	Expression *in vitro*	Expression *in vivo* (women)	Virulence impacts	Distribution
TbpA/TbpB	Fe/Transferrin	NGO1495/ NGO1496	NMB0461/ NMB0460	NEIS1690/ NEIS1691	100 kDa	Fe repressed	High 4/4	Required for human infection	Most *Neisseriae*
LbpA/LbpB	Fe/Lactoferrin	NGO0260 (truncated)	NMB1540/ NMB1541	NEIS1468/ NEIS1469	102 kDa	Fe repressed	Low 3/4	Can substitute for Tf-Fe uptake system in human infection	Gene deletion in many Ngo; locus present in most *Neisseriae*
HpuA/HpuB	Heme/ Hemoglobin	NGO2110/ NGO2109	None	NEIS1946/ NEIS1947	90 kDa	Fe repressed	Low 2/4	Phase 'on' during first half of menstrual cycle in infected women	Most *Neisseriae*; phase variable
FetA	Fe/catecholate Siderophores	NGO2093	NMB1988	NEIS1963	79 kDa	Fe repressed	2/4	None known	All *Neisseriae*; phase variable
TdfF	Fe?	NGO0021	NMB1882	NEIS0338	80 kDa	Fe repressed[d]	No	Contributes to intracellular survival	Only in pathogenic *Neisseriae*
TdfG	Unknown	NGO0553	None	NEIS2646	136 kDa	Fe repressed	Low 2/4	None known	Only in Ngo and *N. elongata*
TdfH	Zn/Calprotectin	NGO0952	NMB1497 (CbpA)	NEIS1428	104 kDa	Zn repressed	4/4	Important for NET survival	Most *Neisseriae*
TdfJ	Zn/?	NGO1205	NMB0964 (ZnuD)	NEIS0944	86 kDa	Fe induced; Zn repressed	4/4	Facilitates systemic Nme infection in mice	All *Neisseriae*
FbpA	Fe	NGO0217	NMB0634	NEIS0578	35 kDa	Fe repressed	High 4/4	Decreased Fe uptake from Tf/Lf	Most *Neisseriae*
FbpB	Fe	NGO0216	NMB0633	NEIS0577	56 kDa	Fe repressed	High 4/4	Decreased Fe uptake from Tf/Lf	Most *Neisseriae*
FbpC	Fe	NGO0215	NMB0632	NEIS0576	38 kDa	Fe repressed	High 4/4	Decreased Fe uptake from Tf/Lf	Most *Neisseriae*
ZnuC (MntA)	Zn	NGO0170	NMB0588	NEIS530	28 kDa	Zn repressed	High 2/4	Contributes to intracellular survival and resistance to oxidative stress	Most *Neisseriae*
ZnuB (MntB)	Zn	NGO0169	NMB0587	NEIS0529	29 kDa	Zn repressed	3/4	Contributes to intracellular survival and resistance to oxidative stress	Most *Neisseriae*
ZnuA (MntC)	Zn	NGO0168	NMB0586	NEIS528	34 kDa	Zn repressed	High 4/4	Contributes to intracellular survival and resistance to oxidative stress	Most *Neisseriae*
TonB	Energization system	NGO1379	NMB1730	NEIS1650	29 kDa	Iron repressed	High 4/4	Impaired metal uptake	All *Neisseriae*
ExbB	Energization system	NGO1378	NMB1729	NEIS1649	26 kDa	Iron repressed	High 4/4	Impaired metal uptake	All *Neisseriae*
ExbD	Energization system	NGO1377	NMB1728	NEIS1648	15 kDa	Iron repressed	High 4/4	Impaired metal uptake	All *Neisseriae*

[a] *N. gonorrhoeae*
[b] *N. meningitidis*
[c] Molecular weight
[d] Not expressed in bacteriological medium.

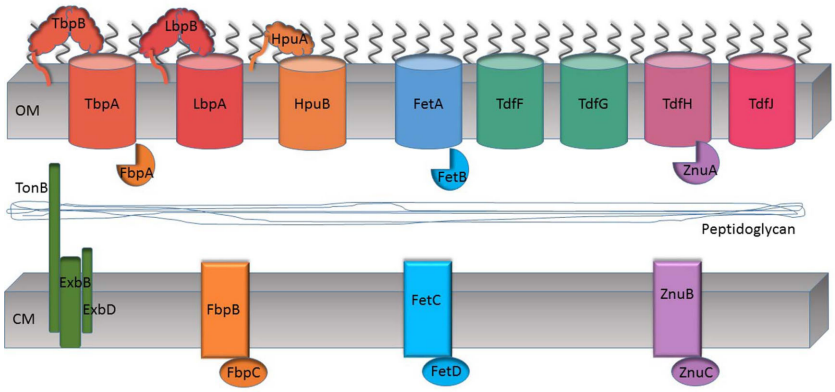

Figure 2. TonB-dependent transporters and ABC transport systems produced by the pathogenic *Neisseria* species. Barrel shapes in the outer membrane (OM) are meant to represent the eight TonB-dependent transporters produced by the *Neisseriae*. Three systems, Tbp, Lbp and Hpu, also include a lipoprotein component (TbpB, LbpB and HpuA, respectively), which are shown tethered to the outer membrane by a lipid moiety. Ligands for each system are described in the text. Black lines extending from the outer membrane are meant to represent lipooligosaccharide. The peptidoglycan layer is shown in the periplasm. The TonB, ExbB and ExpD proteins are shown tethered to or integral in the cytoplasmic membrane (CM). This system energizes transport through the outer membrane by the TonB-dependent transporters. Three ABC transport systems are also represented. FbpABC transports ferric iron; FetBCD transports catecholate siderophores; ZnuABC (sometimes referred to as MntABC in the literature) transports zinc and manganese.

between promoter elements resulted in maximal expression, whereas 16 nucleotides resulted in greatly diminished expression. The rate of variation was measured as $\sim 1 \times 10^{-2}$ in gonococcal strain FA1090 (Carson *et al.* 2000). While the *fet* genes are present in all *Neisseriae* genomes (Marri *et al.* 2010), the degree to which the genes are expressed depends therefore on the number of C residues in the promoter and the Fe condition of the medium, which controls expression through both Fur and MpeR. The FetA protein is highly conserved among members of the *Neisseriae*, with diversity being limited to a hypervariable region in an extracellular loop (Kortekaas *et al.* 2006, 2007; Saleem *et al.* 2012). This restricted sequence variability does not occlude the Fe-binding pocket but perhaps, due to its antigenicity and variable character, serves as an immunological 'decoy' to attract antibodies that would not interfere with Fe-binding function (Saleem *et al.* 2012). The limited variability in this protein antigen has been used epidemiologically to track meningococcal strains; however, the phase variable nature of the antigen suggests it is not an ideal vaccine target (Thompson, Feavers and Maiden 2003).

The *Neisseria* species subvert nutritional immunity by hijacking host metal scavenging proteins

Iron transporters

While the *Neisseria* species can take advantage of xenosiderophores as Fe sources, they do not rely on this variably present environmental resource. Instead, to survive in the face of nutritional immunity, they encode genes that enable them to directly employ host-specific, Fe-containing proteins as metal sources. The best characterized Fe-uptake system possessed by the pathogenic *Neisseriae* is that which allows uptake of Fe from the host protein transferrin (Tf). Tf is produced by the liver, is the major Fe-transporting protein in the human body and is found on the serum in micromolar concentrations (Weinberg 1975). The neisserial Tf receptor system is absolutely specific for human Tf (hTf) (Cornelissen, Biswas and Sparling 1993) and exclusively binds to and extracts the iron from the C-lobe (Noinaj *et al.* 2012b).

Unlike the more typical siderophore-Fe uptake systems, the Tf receptor system is comprised of two distinct proteins (Table 1; Fig. 2). Resembling the TonB-dependent siderophore transporters, the integral outer membrane component of the Tf-Fe uptake system is TbpA, which is larger than average TonB-dependent transporter at 100 kDa (Cornelissen *et al.* 1992; Legrain *et al.* 1993). The crystal structure of meningococcal TbpA demonstrates that the basic structure of TonB-dependent transporters has been maintained in this larger protein, with the shared topology comprising 22 transmembrane β-strands and an amino terminal plug domain (Noinaj *et al.* 2012b). The additional amino acid residues in TbpA are localized to the 11 surface-exposed loops, resulting in a larger protein-interacting interface on the cell surface. The second component of the Tf-iron uptake system is TbpB, which is a fully surface-exposed protein that is attached to the outer leaflet of the outer membrane via an amino-terminal lipid moiety (Legrain *et al.* 1993; Anderson, Sparling and Cornelissen 1994). Both TbpA and TbpB interact specifically with the C-lobe of hTf (Noinaj *et al.* 2012b); however, TbpB has the additional specificity of interacting solely with the ferrated form of Tf (Cornelissen and Sparling 1996), enabling the receptor system to reel in the only ligand that is employable as an iron source (Noinaj, Buchanan and Cornelissen 2012a). Mutants unable to produce TbpA cannot employ hTf as an iron source (Cornelissen *et al.* 1992; Legrain *et al.* 1993); mutants lacking TbpB internalize Fe from hTf less efficiently (Anderson, Sparling and Cornelissen 1994). The latter phenotype is consistent with the role of TbpB in effectively acquiring the ferrated form of hTf and furthermore disposing of the deferrated ligand once Fe has been extracted (Noinaj, Buchanan and Cornelissen 2012a). Relevant to vaccine-development efforts (see below), the Tf-iron acquisition system is expressed by all *N. gonorrhoeae* and *N. meningitidis* strains, is not subject to phase or antigenic variation and is critical for the survival of the gonococcus in a human experimental infection model (Cornelissen *et al.* 1998).

The *tbp* genes are encoded by a bi-cistronic operon (Ronpirin, Jerse and Cornelissen 2001) with the *tbpB* gene upstream of the *tbpA* gene (Anderson, Sparling and Cornelissen 1994). The genes are regulated by the Fe-sensitive Fur protein, resulting in iron abundance repressing expression of the genes (Velez Acevedo *et al.* 2014). In addition, the genes are expressed such that *tbpB* transcripts outnumber *tbpA* transcripts by approximately

2:1 (Ronpirin, Jerse and Cornelissen 2001). Moreover, we have recently demonstrated that a very long, apparently untranslated RNA species is encoded upstream of *tbpB*, interruption of which leads to changes in *tbp* gene expression and stoichiometry (Velez Acevedo *et al*. 2014). In addition to being iron regulated by the Fur repressor, expression of the *tbp* operon is further controlled by the response regulator MisR of the two-component regulatory system, which also includes the sensor kinase MisS (Kandler *et al*. 2016).

All *N. meningitidis* and some *N. gonorrhoeae* strains (Marri *et al*. 2010) also express a closely related Fe uptake system that enables the use of human lactoferrin (hLf) as an iron source. As with the Tf-Fe uptake system, two proteins participate: LbpA and LbpB (Biswas and Sparling 1995; Lewis *et al*. 1998; Biswas *et al*. 1999; Prinz *et al*. 1999). LbpA is the TonB-dependent transporter component and LbpB is the lipid-modified surface-exposed component (Table 1; Fig. 2). Less detailed structural or functional information is known about this system. Unlike the Tf-Fe uptake system, the *lbp* genes are subject to phase variation by virtue of a slipped strand in the upstream gene in the operon (*lbpB*) (Biswas *et al*. 1999). Approximately half of gonococci do not possess the genes to encode the Lf-Fe uptake system due to a large deletion (Anderson *et al*. 2003). Strains unable to produce the Lbp proteins retain infectivity in experimental human infections and in naturally infected humans as *lbp* mutant strains have been isolated from clinical gonorrhea cases (strain FA1090, for example) (Jerse 1999). Nevertheless, a genetically engineered strain that was unable to employ hTf but was able to use hLf as an Fe source was created and shown to be infectious in the human experimental gonorrhea model, indicating that production of the Lbp proteins can substitute for the artificial loss of the Tbp proteins (Anderson *et al*. 2003).

Two systems enable the *Neisseriae* to variously utilize protein-bound heme moieties. All gonococci have the capacity to produce the two-component hemoglobin utilization system comprised of HpuA and HpuB (Chen *et al*. 1996; Chen, Elkins and Sparling 1998) (Table 1; Fig. 2). Unfortunately, the nomenclature is reversed for this system in that the *hpuA* gene lies upstream and encodes the lipoprotein whereas the *hpuB* gene lies downstream and encodes the TonB-dependent transporter (Lewis *et al*. 1997). This system is also expressed by some meningococci and enables *N. meningitidis* to utilize hemoglobin bound to haptoglobin (Lewis *et al*. 1998). In both *Neisseria* species, the *hpuAB* system is phase variable due to slipped-strand mispairing (Chen, Elkins and Sparling 1998; Lewis *et al*. 1999). *In vivo*, the only variants isolated from humans with gonococcal infections are phase off for the *hpu* system, with the exception of infected women in the first half of their menstrual cycle (Anderson *et al*. 2001). The latter observation suggests that the presence of hemoglobin in menstrual blood selects for variants that can produce this transport system.

The second hemoglobin-heme transport system is encoded by a single protein, produced only by some *N. meningitidis* strains (Richardson and Stojiljkovic 1999). HmbR is a TonB-dependent transporter, similar to TbpA, LbpA and HpuB, but in this case functions alone to enable some meningococcal strains to use hemoglobin as an iron source. The *hmbR* gene is present as a frame-shifted pseudogene in *N. gonorrhoeae* genomes (Harrison *et al*. 2013). Highly virulent strains of *N. meningitidis* tend to possess both *hpuAB* and *hmbR* genes, or *hmbR* alone, suggesting hemoglobin utilization correlates with increased virulence (Tauseef *et al*. 2011; Lucidarme *et al*. 2013). Like the *hpuAB* genes, the meningococcal *hmbR* locus is phase variable by slipped-strand mispairing (Richardson and Stojiljkovic 1999). An *hmbR* mutant of *N. meningitidis* was not attenuated for growth in human blood, perhaps due to the production of the Tbps, which enables the use of hTf as a sole iron source in whole blood (Bidmos *et al*. 2015).

Zinc transporters

As shown in Table 2 and Fig. 2, the pathogenic *Neisseria* species encode two outer membrane transporters characterized as Zn importers. Turner *et al*. (2001) first identified three TonB-dependent transporters of unknown function in the FA1090 genome database and gave them the appellations *tdf* for TonB-dependent function. It was surmised at the time that these transporters would enable the gonococcus to import other forms of Fe. However, we recently demonstrated that both TdfH (Turner *et al*. 2001) and TdfJ (Cornelissen and Hollander 2011) (Tables 1 and 2) enable the gonococcus to import Zn (Jean *et al*. 2016). Furthermore, production of TdfH enabled the gonococcus to bind to calprotectin (CP) and deliver the payload of Zn into the gonococcal cell (Jean *et al*. 2016). We showed that TdfH production was Zn repressed in a Zur-dependent fashion and that expression of *tdfH* was MisRS regulated but not Fur regulated (Jean *et al*. 2016). A similar ligand specificity and regulatory network was demonstrated for the meningococcal homologue of TdfH, which was renamed CbpA for CP-binding protein A (Stork *et al*. 2013).

A second TonB dependent transporter, TdfJ, is expressed by all *Neisseriae* (Table 1). Kumar, Sannigrahi and Tzeng (2012) showed that the meningococcal homologue of TdfJ contained a consensus-like hemin-binding motif and did in fact sediment heme when overexpressed by *E. coli*. TdfJ and its meningococcal homologue are both Zn repressed, and we recently demonstrated that TdfJ, similar to TdfH, enables the gonococcus to accumulate Zn, but in this case Zn uptake is not enabled by the presence of CP (Jean *et al*. 2016). Unlike its meningococcal homologue, TdfJ is iron induced (Jean *et al*. 2016). The meningococcal homologue was recently crystallized by Calmettes *et al*. (2015) and strikingly the structures generated contained Zn even though additional metals were not added to the expression or crystallization protocols. Due to its contribution to Zn-dependent growth of *N. meningitidis*, this protein was renamed ZnuD by Stork *et al*. (2010). ZnuD has been postulated to be a potential vaccine candidate due to the fact that it elicited bactericidal antibodies against *N. meningitidis* and it is well conserved across the *Neisseriae* (Stork *et al*. 2010).

Other poorly characterized TonB-dependent, outer membrane transporters

Two other TonB-dependent transporters, TdfF and TdfG (Table 1; Fig. 1), are associated with Fe, but have been incompletely characterized. The gene encoding TdfF is present in all pathogenic *Neisseriae* strains but in none of the commensal *Neisseriae* (Marri *et al*. 2010), suggesting it plays an important role in pathogenesis. We demonstrated that production of TdfF was not detectable in bacteriological growth medium but could be detected by Western blot if gonococci were grown in epithelial cell culture media (Hagen and Cornelissen 2006). Expression of TdfF under these conditions was iron repressed. We furthermore demonstrated that a mutant unable to express *tdfF* was attenuated for growth within epithelial cells and this growth defect was reversed upon addition of supplemental Fe, suggesting that TdfF is an Fe transporter for metal accessed by the gonococcus inside of epithelial cells (Hagen and Cornelissen 2006). We have not yet identified a ligand for TdfF. Intracellular sources of Fe that could possibly be

Table 2. Comparison of Zn transporters produced by *N. gonorrhoeae* (Ngo) and *N. meningitidis* (Nme).

Name		Ligand/metal		Zur regulation		Fur regulation		Other observations	
Ngo	Nme	Ngo	Nme	Ngo	Nme	Ngo	Nme	Ngo	Nme
TdfH	CbpA[a]	Cp/Zn[b]	Cp/Zn[a]	Yes[b]	Yes[c,a]	No[b]	No[d]	Regulated by MisRS[b,e]	Regulated by MisRS[a]
TdfJ	ZnuD[f]	Zn[g,b]	Zn[g,f,h]	Yes[b]	Yes[c,f]	Yes[b] (iron activated)	No[d]	Contains consensus heme-binding motif	Elicits bactericidal antibodies[f]

[a]Stork *et al.* (2013)
[b]Jean *et al.* (2016)
[c]Pawlik *et al.* (2012)
[d]Grifantini *et al.* (2003)
[e]Regulation only demonstrated in gonococcal strain FA19, not in FA1090.
[f]Stork *et al.* (2010)
[g]Unknown whether free or protein-complexed Zn is ligand for TdfJ.
[h]Calmettes *et al.* (2015)

accessed by the *Neisseriae* include ferritin, Fe chelated to organic acids or to glutathione, Tf, Lf, heme and hemoglobin. Of these, only ferritin has been demonstrated to contribute to the intracellular survival of *N. meningitidis* (Larson, Howie and So 2004). Intracellular replication of *N. meningitidis* was demonstrated to be TonB-dependent (Larson *et al.* 2002) and to result in degradation of ferritin (Larson, Howie and So 2004). Heme, hemoglobin, Tf and Lf did not contribute to intracellular survival based upon the replication of mutants unable to transport Fe from these ligands. Thus, it is conceivable that TdfF is the receptor that recognizes Fe derived from degraded ferritin and internalizes the metal in a TonB-dependent fashion, although the link between ferritin degradation and TdfF-dependent Fe uptake has not been directly demonstrated in either pathogenic species of *Neisseriae*.

TdfG is also Fe repressed (Turner *et al.* 2001), but similarly, no ligand has been identified for this TonB-dependent transporter. This protein is expressed nearly exclusively by *N. gonorrhoeae* strains, and no other *Neisseriae* (Marri *et al.* 2010), and is the largest predicted TonB-dependent transporter, at 136 kDa.

ABC transporters for metals

After traversing the outer membrane, metals are bound by a PBP, which shuttles the chelated metal across the periplasm and subsequently docks with the cognate cytoplasmic permease. The plasma membrane transporter is energized by ATP hydrolysis, enabling the metal to traverse the cytoplasmic membrane and enter the cytoplasm. Two transport systems have been well characterized as Fe and Zn uptake systems for the *Neisseriae*: FbpABC (Morse *et al.* 1987; Chen *et al.* 1993) and ZnuABC (Chen and Morse 2001; Lim *et al.* 2008; Stork *et al.* 2010), respectively (Table 1; Fig. 2). The latter system has also been referred to as MntABC. As anticipated, the Fbp system is Fe repressed while the Znu system is Zn repressed. Both systems demonstrate specificity for unchelated metals; however, we demonstrated that FbpA can also contribute to catechol-type siderophore internalization, perhaps by binding strongly to ferric Fe (Strange, Zola and Cornelissen 2011). In strains in which the Fet system is phase off or disabled by point mutation, catechol-type siderophores can still be internalized in an FbpA-dependent manner (Strange, Zola and Cornelissen 2011).

The *Neisseria* species are human-specific pathogens

The genus *Neisseria* includes approximately 20 species, several of which colonize the mucosal membranes of animals (Weyand 2017). However, the pathogenic *Neisseria*, including *N. meningitidis* and *N. gonorrhoeae*, are obligate human pathogens, having no reservoir in the environment or other animals. Having evolved with humans for millennia, the pathogenic *Neisseria* are exquisitely specific for many human proteins and ligands, a phenomenon that contributes to the host-restricted virulence of these pathogens. Given the specificity with which *N. gonorrhoeae* and *N. meningitidis* interact with their human hosts, modeling the diseases caused by these pathogens in animals has been problematic. Non-human primates have been employed for virulence studies (Weyand 2017), but these studies are expensive and fraught with ethical issues. Estradiol-treated female mice have also been employed to study lower genital tract colonization and clearance (Jerse *et al.* 2011), but the host restriction imposed by the specificity of *N. gonorrhoeae* for many human ligands limits the type of experiments that are possible in this model. As an improvement to this mouse model, several transgenes have been added to several different breeds of male and female mice, enhancing the utility of the model by overcoming the host restriction to some degree. The human genes for transferrin (Zarantonelli *et al.* 2007), lactoferrin (Ostan *et al.* 2017), CEACAMs (Buckwalter *et al.* 2017), factor H (Beernink *et al.* 2011; Rossi, Konar and Beernink 2016) and CD46 (Wang *et al.* 2017) have been integrated into the mouse genome by a variety of research groups in efforts to enhance infectivity and evaluate virulence factors.

The human-specific nature of the neisserial hTf interaction (Cornelissen, Biswas and Sparling 1993) was recently considered in an evolutionarily study by Barber and Elde (Barber and Elde 2014). The majority of the most rapidly evolving sites in hTf are in the C-lobe, which is the only lobe to which neisserial TbpA binds. Modest changes in hTf, even those currently present in the great apes, result in an inability of TbpA to recognize this very similar protein. This in turn results in resistance to infection by the pathogenic *Neisseria* and host restriction. These observations reinforce the idea that evolution is ongoing between bacterial pathogen and host, particularly with respect to nutritional immunity. The authors posit that bacterial 'iron piracy'—a term first coined in 1994 in the title of a review of the transferrin-iron acquisition system from *N. gonorrhoeae* (Cornelissen and Sparling 1994)—can be overcome by rapid evolution of mammalian transferrins.

The pathogenic *Neisseria* species employ metal piracy to survive *in vivo*

As shown in Table 1, the majority of known or proposed players in metal transport in the pathogenic *Neisseriae* are expressed

in vivo in women recently infected with *N. gonorrhoeae*. In the study published by McClure *et al.* (2015), gene expression was evaluated by RNA-seq in four women with recently acquired gonorrhea. Low levels of expression were detected for the *lbp* and *hpu* genes. Likewise, expression of *fetA* was only detected in 2/4 women and *tdfG* was also poorly expressed in 2/4 subjects. The only *tdf* transcript listed in Table 1 that was not detected at all in this study was *tdfF*. These results indicate that the lower female genital tract represents a low metal environment and furthermore that the majority of systems documented to facilitate metal uptake are well expressed and anticipated to be critical to survival in this environment. It is unclear at present whether the same is true of men with gonorrhea.

Also shown in Table 1 are virulence impacts of inactivating the metal transport systems in the pathogenic *Neisseria* species. The Tf-Fe acquisition system is expressed by all strains of both *N. gonorrhoeae* and *N. meningitidis*. We tested whether a mutant unable to produce the Tbps was infectious in a human male infection model almost two decades ago (Cornelissen *et al.* 1998). Compared to the parental strain, FA1090, the *tbp* mutant strain was unable to elicit any signs or symptoms of urethritis in the male volunteers inoculated with this strain. The parental strain is naturally unable to utilize Lf as an iron source as a deletion in the genome replaces the *lbp* genes. Thus, the mutant created in our initial infection study was effectively unable to utilize either Lf or Tf. Subsequently, the Sparling group (Anderson *et al.* 2003) replaced the naturally mutant *lbp* locus in FA1090 with the wild-type version from another strain. This resulted in an infectious strain but one that also has an iron phenotype that has never detected in nature. A strain that was Lf+/Tf+ had a competitive advantage over a strain that was Tf+/Lf– (Anderson *et al.* 2003). The conclusion from these studies was that the *lbp* system can substitute for the *tbp* system, but that there is a selective force driving the loss of the *lbp* genes from the *N. gonorrhoeae* genome.

In a related study, *N. gonorrhoeae* samples from naturally infected human subjects were tested for whether or not the Hpu system was expressed (Anderson *et al.* 2001). The Hpu system was phase off in all male subjects and in most female subjects, with the notable exception being in women in the first half of their menstrual cycle. The conclusion drawn from this study was that menses, and abundant hemoglobin, selects for strains that are able to internalize heme from hemoglobin using the Hpu receptor system (Anderson *et al.* 2001). There is epidemiological evidence to suggest that production HmbR by *N. meningitidis* enhances virulence (Tauseef *et al.* 2011; Harrison *et al.* 2013; Lucidarme *et al.* 2013). However, neither the HpuAB system nor the Fet system is necessary to colonize the female mouse genital tract (Jerse *et al.* 2002). In fact, none of the characterized iron transport systems was necessary in the Balb/C, estradiol-treated mouse model of colonization (Jerse *et al.* 2002). However, since neither hTf nor hLf are present this mouse model, the absence of dedicated receptors to internalize Fe from these proteins would not be anticipated to be detrimental for *N. gonorrhoeae* survival in this model.

Although gonococcal mutants unable to produce TdfH or TdfJ have not been tested for infectivity in humans, we recently demonstrated that TdfH is important for survival in the presence of NETs (Jean *et al.* 2016). This defect can be overcome by supplementation with Zn and is presumably due to sequestration of Zn by Cp in NETs, which is overcome by the production of TdfH by *N. gonorrhoeae*. A mutant unable to produce the TdfJ homologue, ZnuD, was shown by Calmettes *et al.* (2015) to be defective for systemic infection by *N. meningitidis* in mice; however, localized infection of the nasopharynx was unimpaired in the mutant. This suggests that the ligand and/or key virulence function for TdfJ resides in the bloodstream.

While the Ton system, which energizes all TonB-dependent transporters, has not been evaluated for its virulence impacts in humans, one would expect that the inability to sequester metals at high affinity would result in decreased virulence *in vivo*. The fact that the Ton system is highly expressed *in vivo* (McClure *et al.* 2015) is consistent with this proposition. The ABC transport system that facilitates import of ferric iron (FbpABC) has likewise not been tested for *in vivo* virulence impacts, but is anticipated to be critical for growth and survival in humans, which is a low iron environment. Finally, the ZnuABC system has also not been tested for virulence defects in humans either, but a mutant unable to produce any of these proteins was found to be defective in intracellular survival (Lim *et al.* 2008) and was hypersensitive to oxidative stress (Tseng *et al.* 2001). These *in vitro* phenotypes suggest that the ZnuABC system plays an important role *in vivo* as well.

Given the exquisite species specificity of the metal transport systems employed by the pathogenic *Neisseria* species, testing for virulence defects will result in the most relevant answers if conducted in humans. The human male infection model has great utility in this regard, but is limited in that only the initial phase of infection can be examined before human subjects must be treated with antibiotics. In addition, similar infection experiments cannot ethically be conducted in women and it is conceivable that some virulence factors could be gender specific in their importance to infection. Obviously, *N. meningitidis* infections could not be experimentally conducted with human subjects, and thus animal models must suffice. A promising advance related to animal modeling is the facility with which human transgenes can be introduced into the mouse genome. Expressing human CEACAMs, hTf and hLF in mice has recently enabled researchers to better model infection caused by these human-specific pathogens.

Sabotaging metal piracy systems could lead the way towards better therapies and prevention of diseases caused by the pathogenic *Neisseria*

Iron acquisition pathways, since they are critically important for growth and survival of most bacteria, have long been attractive candidates for development of efficacious vaccines or therapies. Recently, the yersiniabactin receptor has been shown to be a viable vaccine target in the prevention of urinary tract infections (UTIs) caused by *E. coli* (Brumbaugh, Smith and Mobley 2013). Similarly, vaccination with yersiniabactin or aerobactin, individually conjugated to proteins, demonstrated protective effects against *E. coli* in a mouse model of UTI (Mike *et al.* 2016).

Two approaches have been considered when developing therapeutic agents targeting metal acquisition pathways. The first and oldest is to link siderophores to antibiotics. These so-called sideromycins directly link a metal-binding moiety to an antimicrobial agent. Several naturally occurring sideromycins are known, including the albomycins, produced by actinomycetes, which link a ferrichrome-type siderophore to a protein synthesis inhibitor (Ji, Juarez-Hernandez and Miller 2012). This 'Trojan Horse' approach has been coopted by researchers to design and biochemically synthesize antimicrobials that similarly 'trick' the bacterial pathogen into internalizing the inhibitor by virtue of the iron-binding moiety. Examples include the *Pseudomonas* siderophore pyoverdin conjugated to ampicillin or cephalexin (Ji, Juarez-Hernandez and Miller 2012). Additionally, pyochelin,

another *Pseudomonas* siderophore, has been conjugated to fluoroquinolones (Ji, Juarez-Hernandez and Miller 2012). This approach has been applied to siderophores produced by other pathogens as well, including *Plasmodium falciparum*, *M. tuberculosis* and *Acinetobacter baumanii* (Ghosh *et al.* 2017). The second approach is to target siderophore biosynthetic pathways using small molecule inhibitors (Lamb 2015). Interference with different steps in bacterial siderophore biosynthetic pathways is being considered as therapy against *M. tuberculosis* and *Y. pestis* (Ferreras *et al.* 2005). Finally, small molecule inhibitors of heme utilization enzyme, HemO, have shown therapeutic activity against *P. aeruginosa* (Hom *et al.* 2013).

Given the precedent from other pathogens, and the fact that the neisserial metal transporters are conserved, largely expressed *in vivo* and, where tested, important for virulence, these systems make attractive vaccine and drug targets for these bacterial pathogens. *Neisseria gonorrhoeae* in particular is currently impossible to prevent by vaccination and becoming increasingly difficult to treat. The Tf-iron acquisition system appears the most tractable target since it is expressed by all strains, not subject to phase or antigenic variation and critical for gonococcal survival *in vivo*. Sequence conservation, particularly in TbpA, suggests that a vaccine composed of this protein could potentially be cross-reactive among strains, or even cross-protective against *N. meningitidis* strains (Petousis-Harris *et al.* 2017). It is conceivable that targeting only one metal transport system would allow growth and survival if one or more of the other metal transport systems remains functional. With respect to vaccine development, this concern is only valid if the only correlate of protection is blocking of ligand binding and metal uptake. If vaccination also results in other protective effects, such as fixing complement, opsonophagocytosis or aggregation and clearance, then blocking metal import in addition to these protective effects would simply be icing on the cake. We have demonstrated that vaccination of Balb/C mice (without supplementation or expression of hTf) with the gonococcal Tbps resulted in potentially protective activities, including production of serum antibodies that fixed complement and development of mucosal secretions that blocked hTf-Fe utilization by gonococci grown *in vitro* (Price, Russell and Cornelissen 2005; Price *et al.* 2007). Meningococcal Tbps are similarly able to elicit bactericidal antibodies in the serum of immunized mice (Rokbi *et al.* 1997). These observations in non-transgenic mice suggest that these proteins could be important components in an efficacious vaccine to prevent gonorrhea. With respect to treatment, crystal structures for the Tbps can guide small molecule screens for compounds that interfere with Tf-Fe utilization. As with the vaccination approach, targeting a single metal uptake system may be insufficient, but the same crystal structure based approach could be deployed against other metal uptake systems to ultimately identify cocktail treatment regimens that target multiple aspects of neisserial metal import. It is anticipated that Zn and other metal acquisition systems are equally as feasible as vaccine and drug targets as those pathways involved in Fe uptake.

CONCLUSIONS AND PERSPECTIVES

The human host presents a daunting environmental challenge for bacterial pathogens. Transition metals, necessary for a variety of biochemical reactions and energy generation, are aggressively sequestered by the host in an attempt to inhibit microbial growth and survival. Human proteins including transferrin, lactoferrin and calprotectin function as components of the host's armamentarium against invading pathogens, who must defend against 'nutritional immunity' in order to survive. Against this backdrop, bacteria have evolved a variety of metal sequestration strategies of their own; the ongoing evolutionary struggle continues to play out as the invader develops ever-more sophisticated strategies against the host.

While most bacteria have overcome this sequestration challenge by synthesizing and deploying siderophores for metal solubilization, chelation and transport, the pathogenic *Neisseria* species resort instead exclusively to 'iron piracy'. These ultimate kleptomaniacs acquire the necessary metals directly from their host, by producing surface receptors dedicated to binding to and removing the metals from human metal-binding proteins. They can also steal Fe-siderophores from bacteria in the community, making them effective societal 'cheaters'. The specificity of the pathogenic *Neisseriae* for human metal-binding proteins is hypothesized to at least contribute to, if not be responsible for, the strict host restriction of these pathogens.

The urgent need for vaccines to prevent neisserial diseases, and for better therapies to treat the afflictions caused by these pathogens, prompts us to consider whether interference with the sophisticated metal acquisition systems deployed for their *in vivo* survival could be translated into efficacious preventative and treatment strategies. Interference in Fe acquisition and homeostasis has proven successful in this regard for prevention of and therapy for other bacterial infectious diseases. Rational design of metal-import system based vaccines and therapeutics begins with complete crystal structures, a few of which are currently available. Outstanding questions remain however, including what routes of delivery and adjuvants induce the most effective immune responses, which antigens result in the most cross-reactive responses, and can protein-based vaccine components be employed to protect against diseases caused by *both* pathogenic *Neisseria* species? With respect to potential therapies, which metal transport systems are the most 'druggable', would these therapies be effective against *Neisseria* species only, and will interference with a single metal import system allow for bypass of the defect by upregulating or turning on another system *in vivo*? Vigorous, collaborative research in the next decade will hopefully be able to answer these outstanding questions and could potentially lead to novel prevention and treatment strategies that can be deployed against these very effective human-specific pathogens.

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