MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, Mar. 2004, p. 154–171 Vol. 68, No. 1
1092-2172/04/$08.00+0 DOI: 10.1128/MMBR.68.1.154–171.2004
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Iron Transport Systems in *Neisseria meningitidis*†

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INTRODUCTION ... **154**
IRON AVAILABILITY IN THE HUMAN HOST ... **155**
IRON ACQUISITION MECHANISMS ... **156**
Transferrin and Lactoferrin Receptors ... **156**
Genetic arrangement and regulation by Fur ... **157**
Structure predictions ... **158**
Structure and functional relationships ... **158**
Energy from the TonB-ExBD system ... **159**
Transport through the periplasm ... **159**
Ferric iron in the cytoplasm ... **160**
Hemoglobin Receptor HMBR ... **160**
Genetic arrangement and regulation by Fur ... **161**
Structure predictions ... **161**
Structure and functional relationships ... **161**
Energy from the TonB-ExbBD system ... **161**
Transport through the periplasm ... **162**
Heme in the cytoplasm ... **162**
Haptoglobin-Hemoglobin Receptor HPUAB ... **163**
Genetic arrangement and regulation by Fur ... **163**
Structure predictions ... **163**
Structure and functional relationships ... **164**
Energy from the TonB-ExbBD system ... **165**
Transport through the periplasm ... **165**
Other Potential Iron Transporters ... **165**
Vaccine Potential and Trials ... **165**
CONCLUDING REMARKS ... **166**
ACKNOWLEDGMENTS ... **167**
REFERENCES ... **167**

INTRODUCTION

Success, in terms of bacterial survival inside the host, depends on an organism's ability to scavenge essential nutrients. Acquisition of iron and iron complexes has long been recognized as a major determinant in the pathogenesis of *Neisseria meningitidis*. This ability to acquire iron under iron-restricted conditions has undoubtedly contributed to its success as one of the leading causes of bacterial meningitis in children and healthy adults in the world.

N. meningitidis is the organism responsible for two serious human diseases, pyogenic meningitis and meningococcal septicemia. These diseases are secondary infections of the meninges, skin, and other body parts, resulting from systemic spread of meningococci (10). The progression of illness begins with exposure, followed by carriage or colonization, with an occasional advance to the disease state. The carriage rate in normal healthy adults is roughly 10%, and several factors, including the virulence of the strain, host susceptibility, and environmental influences play a role in the development of invasive disease (10). Despite a low risk of contracting meningococcal disease, it remains a serious health problem worldwide. The prevalence of meningococcal disease varies with geographical location, with endemic disease occurring in developed countries and epidemic disease predominating in developing countries, such as the region of sub-Saharan Africa (100, 147). Three factors contribute to its ominous reputation; these include a high mortality rate, a rapid onset of severe disease symptoms, and the potential for epidemic spread.

Research has well established the dependence of *N. meningitidis* growth on iron availability. There are numerous examples of pathogenic bacteria, including *N. meningitidis*, whose virulence is enhanced in experimental infections by the injection of iron compounds into the animal host (see reference 65 for a review). The importance of iron uptake for virulence in *N. meningitidis* was first proposed by Payne and Finkelstein (110). Shortly thereafter, the detrimental effects of iron deprivation on the growth of *N. meningitidis* were described by Archibald and DeVoe (14). These effects included both reduced replication and reduced respiration rates. Iron uptake was also shown to be a crucial factor in meningococcal infection by Holbein et al., who injected iron compounds (iron dextran or human fer-

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† D.P.-B. and M.R.-G. dedicate this paper to the memory of Igor Stojiljkovic (1959-2003).

ritransferrin) into mice and challenged them with a subsequent intraperitoneal inoculation of live *N. meningitidis* (69–71). This resulted in lethal infection, whereas mice injected with only *N. meningitidis* suffered a transient bacteremia and recovered quickly. Iron-starved meningococci grown in vitro were also found to have increased virulence in vivo (34). A hypoferremic response, which reduces the amount of free transferrin by as much as 70%, helps control meningococcal infection in mice (and humans) (168). However, this response is ineffective if the iron concentration is artificially raised as in Holbein's experiments (70, 168).

IRON AVAILABILITY IN THE HUMAN HOST

The majority of iron in the body is stored intracellularly in ferritin (up to 4,500 Fe^{3+} atoms per ferritin molecule) and hemoglobin (57). Extracellular iron found in body fluids is attached to high-affinity iron-binding proteins, such as transferrin in serum and lymph and lactoferrin in milk and secretions. In order for *N. meningitidis* to cause disease, it has to survive in various environments within the host, including the oropharynx, bloodstream, and cerebrospinal fluid, which contain various forms and concentrations of free and complexed iron. Of these environments, the cerebrospinal fluid has the highest content of free iron, at 2.2 μmol/liter (57). The major source of iron available to the meningococci, therefore, is iron complexed to host iron-binding proteins. These host iron-binding proteins have high binding constants for iron, ranging around 10^{36}, and they are usually only 30 to 40% saturated. This correlates to virtually zero free iron for bacterial growth (67). *N. meningitidis* has managed to overcome this iron limitation through the evolution of iron acquisition systems which enable it to use transferrin, lactoferrin, hemoglobin, and haptoglobin-hemoglobin as iron sources (55, 95, 96, 145).

The majority of transferrin is synthesized in the liver and circulates in human serum, functioning to sequester iron. This prevents both the damaging effects of free iron and bacterial growth (57). Transferrin can bind ferric iron at locations of absorption and storage and transport it to locations of utilization. Mammalian cells receive iron from transferrin through a receptor-mediated process involving the internalization of transferrin and the release of iron in endosomal compartments.

Human transferrin is a monomeric 80-kDa bilobed glycoprotein having a ferric ion-binding site and bicarbonate anion-binding site in each lobe (57, 145, 167). All members of the transferrin family have the bilobed structure, with each lobe subdivided into two α/β domains that are separated by a deep cleft that contains the iron-binding site. The proposed mechanism of iron binding has come from crystal studies of rabbit serum transferrin (16). Four amino acids (D63, Y95, Y188, and H249) and two oxygen atoms from the bicarbonate anion function to coordinate the ferric ion in the binding pocket (16, 57). The binding of the anion is believed to neutralize the charges around the site that might repel the ferric ion and to provide the remainder of the six ligands needed for binding (see reference 57 for a review). The mechanism of iron release from transferrin has not been described, but it is believed to involve a conformational change mediated by a hinge-like region that is located behind the iron-binding site and between the two domains. Domain opening may be promoted by a specific receptor protein, reduction of Fe^{3+} to Fe^{2+}, or lowered pH (16).

Transferrin is not an abundant source of iron on mucosal surfaces, although, transferrin receptors have been found in all clinical isolates of *N. meningitidis*. A transferrin receptor-deficient gonococcal strain is avirulent in human model studies, while the parental strain is virulent (47). This demonstrates the importance of the transferrin receptor in *Neisseria gonorrhoeae* infection and indicates that the genitourinary mucosal surface has enough transferrin to support bacterial growth. Because the most abundant source of transferrin is human serum, this substrate is likely to be an important source of iron during the invasive phase of meningococcal infection. However, studies with the gonococcus suggest that transferrin may be an important source of iron on mucosal surfaces as well.

Lactoferrin, a member of the transferrin family of proteins, is found mostly in phagocytic cells and in secretions such as milk, mucus, and tears. Neutrophils actively release lactoferrin at sites of infection (139). The structural and functional features of lactoferrin are very similar to those of transferrin, including its tight yet reversible binding to ferric iron, a conserved three-dimensional structure, and almost identical iron-binding sites (7, 17, 150). The sequence identity between lactoferrins and transferrins is roughly 60% (17). There are, however, unique differences between these related proteins, including a higher affinity of lactoferrin for iron (especially evident at low pH) and bactericidal activity associated with lactoferrin (17). Lactoferrin also has some additional properties that are derived from its surface characteristics, including the ability to bind anionic molecules such as DNA, heparin, and glycosaminoglycans. It can also bind to certain cells and modulate their activity, act proteolytically on certain substrates, and enter cells via nonconventional routes (16). All meningococcal strains tested have been shown to utilize lactoferrin, and supplemental lactoferrin can enhance the virulence of meningococcal infection in mice (95, 140). Utilization of lactoferrin is not essential for causing gonococcal colonization or invasive disease (47). However, recent studies of human gonococcal infection indicate that in the absence of the transferrin receptor, expression of the lactoferrin receptor is sufficient for initiating infection (8). In addition, the expression of both transferrin and lactoferrin receptors results in a competitive advantage over a strain expressing only the transferrin receptor in mixed infections (8).

One important aspect of transferrin and lactoferrin utilization by the meningococcus is that there is a preference for human transferrin and human lactoferrin. Even though there is considerable homology between the transferrins, such as porcine, bovine, sheep, and ovotransferrin, *N. meningitidis* can discriminate against these non-human-derived transferrins (85, 141, 144). This correlates well with the role of *N. meningitidis* as an obligate human pathogen.

Another source of iron for *N. meningitidis* is hemoglobin. In addition, meningococci are capable of using haptoglobin-hemoglobin and free heme, but not heme-hemopexin or heme-albumin, as sources of iron (55). Hemoglobin is the best characterized of host iron-binding proteins (see reference 53 for a review). Briefly, hemoglobin is the oxygen-binding and transport protein of erythrocytes, functioning to deliver oxygen from areas of high concentration to areas of low concentration

throughout the host. The iron component of hemoglobin is the central atom within a heme moiety. The heme groups of hemoglobin are located in heme-binding clefts within each of four subunits and interact hydrophobically with amino acids in the cleft. The heme iron interacts with six ligands within the iron-binding cleft of hemoglobin: four nitrogen atoms from the porphyrin moiety of heme, the imidazole side chain of a histidine residue, and oxygen, when present. Increased concentration of carbon dioxide in the peripheral tissues, which is rapidly converted to carbonic acid, lowers the pH and favors the release of oxygen as the sixth ligand of the heme iron.

Hemoglobin is not readily available for use by *N. meningitidis* because of compartmentalization within the erythrocytes. Spontaneous hemolysis results in small amounts of hemoglobin being released into normal human serum (80 to 800 nM). However, this free hemoglobin rapidly dissociates into two dimers and is rapidly complexed to circulating haptoglobin and transported to the liver. Like that of transferrin, the level of hemoglobin on the mucosal surface of the oropharynx is normally very low, yet meningococci express two different hemoglobin receptors (156). It is possible that a small amount of local hemolysis would make a large difference in the amount of free hemoglobin and haptoglobin-hemoglobin complexes available on mucosa for bacterial growth (145). In addition, these receptors participate in heme utilization by meningococci (61, 102). Other heme-requiring pathogens have been shown to colonize the oropharynx and survive on the available heme (145). Recently, two additional members of hemoglobin family of proteins have been discovered: neuroglobin, which is expressed predominantly in the brain, and cytoglobin, with strongest expression in heart, stomach, bladder, and small intestine (37). Presently, it is not known whether these hemoglobins can be used as a source of iron or heme for bacteria.

IRON ACQUISITION MECHANISMS

Two general mechanisms of iron acquisition in bacteria have been described: siderophore-mediated iron acquisition by cognate receptors and receptor-mediated iron acquisition from host iron-binding proteins (31–33, 42). One important difference between these two mechanisms of iron acquisition is that siderophores and hemophores are taken into the cell whole, whereas iron and heme must be stripped away from host carrier proteins prior to transport. *N. meningitidis* possess several iron uptake systems that rely on high-affinity receptors for iron-bound host proteins, including transferrin, lactoferrin, and hemoglobin (Fig. 1). Each of these systems is discussed in detail below. In situations where information was not available for *N. meningitidis* iron transport systems, data from experiments using the closely related *N. gonorrhoeae* may have been included, and this is indicated appropriately below; for an excellent review of *N. gonorrhoeae* iron transport see reference 133.

Many enteric bacteria use siderophore-mediated iron uptake, and the characterization of these systems has preceded descriptions of receptor-mediated iron acquisition systems. In *N. meningitidis*, however, no low-molecular-mass compounds that scavenge insoluble ferric iron have been identified (13, 171). Original experiments demonstrating the ability of *N. meningitidis* to use iron from human transferrin demonstrated that transferrin-bound iron could not be used if the transferrin was contained in dialysis tubing; this suggests that even if *N. meningitidis* produces siderophores, they cannot remove iron from transferrin and may not have a significant impact in vivo (15). Studies indicate that meningococci may be able to use heterologous siderophores secreted by other bacteria (136). For some time it has been reported that the gonococci could utilize ferric enterobactin and aerobactin as iron sources (38, 170, 171). Recently in *N. gonorrhoeae*, an outer membrane protein named FetA (formerly FrpB) has demonstrated low binding affinity and transport of ferric enterobactin (21, 38, 54, 113). The binding constant of FetA for enterobactin was much lower than for other enterobactin receptors, and it was therefore proposed that this receptor could interact with high affinity to a yet-unidentified, phenolate siderophore (38). A homologous protein, with 91% identity to gonococcal FetA, has been identified in *N. meningitidis* and presumably functions similarly (113, 165).

Transferrin and Lactoferrin Receptors

Two proteins, transferrin-binding protein A (TbpA) and transferrin-binding protein B (TbpB), function as the transferrin receptor in *N. meningitidis* (44, 49, 63, 142). TbpA and TbpB are induced along with several other proteins in the outer membranes of *N. meningitidis* under iron-restricted conditions (22, 86, 144). Initially, an affinity isolation procedure using biotinylated transferrin was employed to demonstrate the presence of two transferrin-binding proteins in *N. meningitidis* (144). The proteins which bound transferrin were TbpA (formerly Tbp1), which was 98 kDa, and TbpB (formerly Tbp2), which was 68 kDa (66, 104, 143). Among different meningococcal isolates, the molecular masses of TbpA and -B vary, with TbpA ranging from 93 to 98 kDa and the more heterogeneic TbpB varying from 68 to 85 kDa (58, 66, 143). TbpA can be found on all strains, both invasive and carrier, and depending on the strain, there are roughly 700 to 4,700 copies per cell (117).

Although it has not been characterized as well as the transferrin receptor, the lactoferrin receptor is believed to be an important virulence factor of the meningococci. The entry site of *N. meningitidis* into the body is the nasopharynx, where lactoferrin predominates as the main source of iron. Additionally, lactoferrin has been shown to cross the blood-brain barrier during acute inflammation and may also serve as a source of iron during the invasive phase of infection (73, 115). Supporting this role, the meningococcal lactoferrin receptor has been found on all strains tested to date (95, 115). In contrast, the gonococcal lactoferrin receptor has been found on only half of clinical isolates (8, 95). This difference may be related to the differences in the niches they occupy, with lactoferrin utilization being an essential function of the meningococci.

The lactoferrin receptor of *N. meningitidis*, like the transferrin receptor, consists of two protein components, LbpA and LbpB. Initial experiments using affinity isolation by lactoferrin identified a 98-kDa lactoferrin-binding protein named LbpA, formerly known as IroA (112, 123, 143). Insertional inactivation of *lbpA* in *N. meningitidis* eliminated lactoferrin binding,

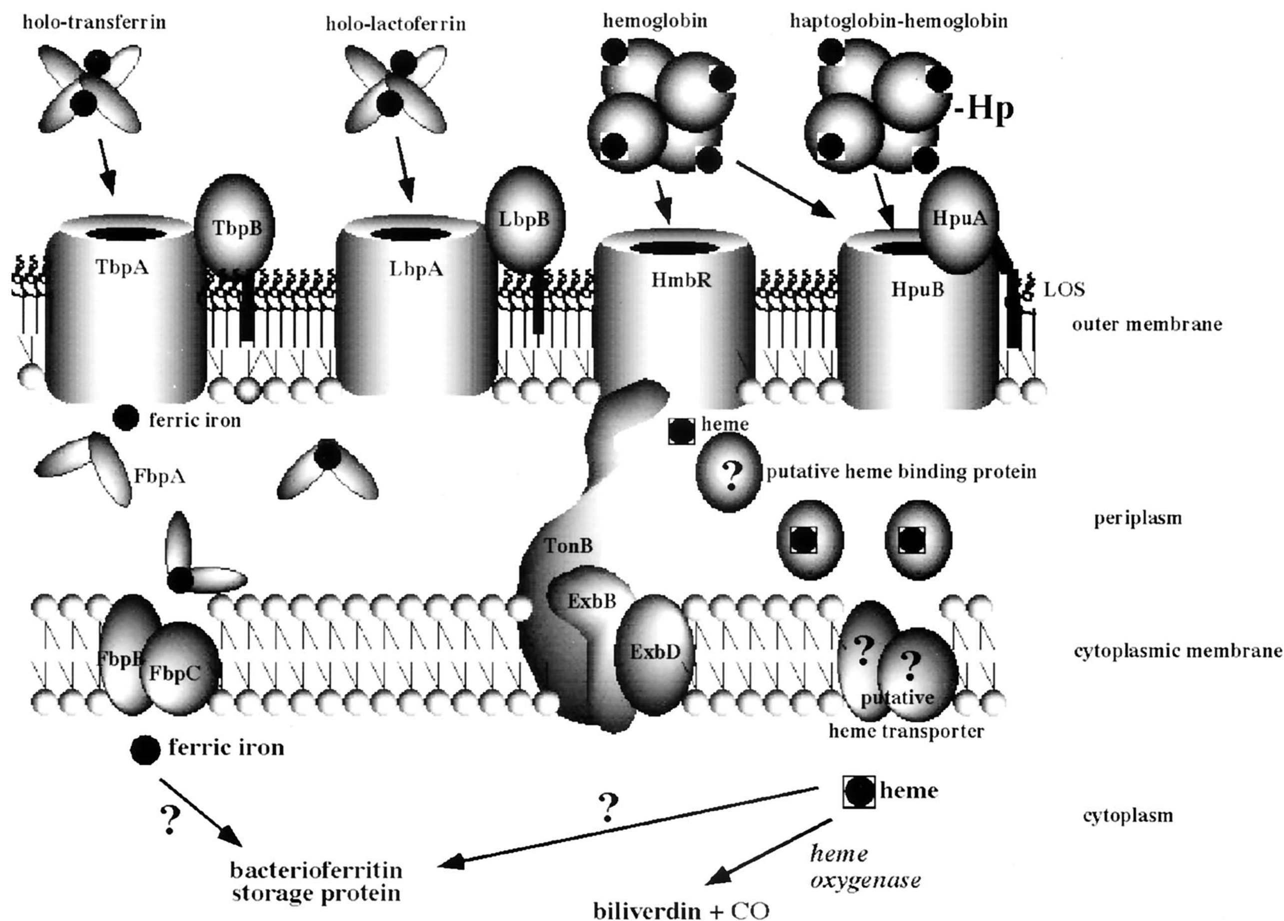

FIG. 1. Schematic representation of iron acquisition systems in *N. meningitidis*. Under iron-limiting conditions, meningococci express several proteins involved in the uptake and processing of iron and heme. Several of these are outer membrane receptor proteins, which recognize transferrin, lactoferrin, hemoglobin, and hemoglobin-haptoglobin. Depicted are the TonB-dependent receptors, TbpAB, LbpAB, HmbR, and HpuAB. All consist of a pore-forming membrane protein and, except for HmbR, a lipoprotein that is involved in substrate binding. An uncharacterized TonB-independent heme uptake system is not shown. The FbpABC transport proteins are involved in the periplasmic transport of ferric iron, and an undescribed system may exist for the periplasmic transport of heme. In the cytoplasm, ferric iron and heme can be stored in bacterioferritin. Heme can also be converted to biliverdin and CO in the cytoplasm by heme oxygenase. Dimeric forms of TonB and the FbpBC proteins from *E. coli* have been described but are not depicted as such here. LOS, lipooligosaccharide

lactoferrin utilization, and expression of the 98-kDa outer membrane protein (23, 123). Under lower-stringency isolation conditions, a second 84-kDa lactoferrin-binding protein was identified and named LbpB (139).

Genetic arrangement and regulation by Fur. The *tbp* locus is a bicistronic operon located on the chromosome of *N. meningitidis*. It consists of open reading frames *tbpB* and *tbpA*, which are separated by an 87-bp intergenic region (86). Unlike the case for many other genes of *N. meningitidis*, there is no phase variation of the transferrin receptor due to slipped-strand mispairing. Therefore, the transferrin receptor could potentially be expressed at all times under in vivo conditions of iron limitation. A putative promoter containing regulatory regions of significant homology to Fur-binding sites precedes the *tbp* locus (86). The Fur protein is a global repressor protein which negatively regulates iron-controlled genes at the transcriptional level (see reference 68 for a review). Although the Fur-binding site at the *tbp* locus has not been characterized, iron limitation has been shown to be the only signal required for expression of the Tbp receptor (144). Derepression of the transferrin receptor in *N. gonorrhoeae* results from mutating its *fur* gene (160). The *N. meningitidis* Fur homologue has also been identified, but studies involving Fur regulation of the meningococcal *tbp* operon have not been reported (76, 159).

Similarly, the genes for the lactoferrin receptor in *N. meningitidis* are on a transcriptional operon, with *lbpB* located upstream of *lbpA* (24, 90, 115, 139). The existence of an operon was predicted, since there are no −10 and −35 sequences upstream of *lbpA* and the stop and start codons of the genes overlap (115). Primer extension, reverse transcriptase PCR, and insertional inactivation of *lbpB* with a cassette containing transcriptional terminators, which disrupted the expression of both LbpA and LbpB, confirmed that the genes constitute an operon (90, 139). The promoter region directly upstream of *lbpB* also has a predicted Fur-binding sequence, which overlaps the −10 sequence, thus implying that these genes are

regulated by the Fur repressor and iron availability. Western blotting and mRNA analysis have confirmed that transcription and LbpA and -B expression are iron regulated, consistent with the upstream Fur-binding site (90, 115).

Structure predictions. Both Tbp proteins and both Lbp proteins are expressed as precursors carrying amino-terminal signal peptides and are localized to the outer membrane. Initially, the translated sequence of TbpB suggested that it was a lipoprotein, since it has a signal II protease cleavage site and does not appear to have membrane-spanning regions. LbpB shares 33% amino acid identity to TbpB (115). Protease susceptibility assays done on the LpbB homologue from *Moraxella catarrhalis* suggested that the protein is largely exposed at the cell surface (139). Like TbpB, LbpB contains a signal peptidase II recognition sequence with an amino-terminal cysteine residue, which implies that it undergoes lipidation (139). Biochemical studies have confirmed that TbpB has a 20-amino-acid signal sequence that is cleaved, followed by lipidation of the first amino acid (cysteine) of the mature protein (91). More than likely, it is anchored to the outer membrane via its N-terminal lipid (145). The crystal structure of TbpB has not yet been determined, but it is predicted to be a bilobed protein like its transferrin substrate since it contains two transferrin-binding domains and has internal amino acid repeat regions (94, 126). A high-affinity transferrin-binding domain which remains stable after exposure to heat and sodium dodecyl sulfate is located in the amino-terminal domain, and a second transferrin-binding site is located in the carboxyl-terminal domain (45, 126, 166). A unique feature of LbpB, not shared by TbpB, is the presence of two long stretches of acidic amino acid residues. These domains are predicted to be important in the interaction with lactoferrin, which is a positively charged molecule (115).

TbpA and LbpA share 25% amino acid identity and a high degree of similarity (63). They also exhibit low homology to other TonB-dependent transport proteins. The predicted structures of TbpA and LbpA are based on their sequence homology with the ferric siderophore receptors (see reference 42 for a review). Homology to this family of transporters suggests that the ancestral meningococcal transferrin and lactoferrin receptors may have been a single-unit transporter like the siderophore receptors; in fact, homologous single-unit transferrin receptors have recently been described for *Pasteurella multocida* and *Histophilus ovis* (56, 106). The two-dimensional models of TbpA and LbpA depict 22 transmembrane-spanning regions that form an extended β-sheet which is predicted to fold into an amphiphillic β-barrel pore structure similar to the structures of all of the characterized bacterial outer membrane proteins (36, 80). The models predict 11 extracellular loops of various sizes. The sizes of both TbpA and LbpA are roughly 20 kDa greater than those of the siderophore receptors, and they are predicted to have larger surface loops (145). Some of these predicted loops have been examined experimentally (29, 122). For LbpA, the cell surface exposure of some of these predicted loops has been analyzed by using antisera against synthetic LbpA peptides (122). Predicted loops shown to be surface accessible included loop 4 (L4), L5, L7, L10, and L12 (122). Additionally, the amino-terminal ends of these proteins may extend into the pores of the predicted barrel structures from the periplasmic side, similar to the plug domains of the ferric siderophore receptors.

TbpA and TbpB function together in the outer membrane of *N. meningitidis* as a transferrin receptor complex. Mutants deficient in both proteins are unable to bind or utilize iron from transferrin (74). Furthermore, mutants deficient in either TbpA or TbpB are also unable to utilize iron from transferrin, but they have transferrin-binding capabilities (74). In *N. gonorrhoeae*, however, mutants deficient in TbpB alone could utilize iron from transferrin at roughly 20% of wild-type levels, suggesting that TbpA requires the cooperative activity of TbpB to be fully functional (9). Several lines of evidence are consistent with TbpA and TbpB colocalization in the outer membrane, including protease susceptibility assay of TbpB (46, 59, 121). Purification and in vitro experiments further support these results. When transferrin affinity chromatography is used to purify the Tbp proteins, TbpA and TbpB can be isolated together under high-ionic-strength conditions (48). TbpA and -B have been examined by photon correlation spectroscopy, which demonstrated that in vitro, they will form a complex in solution (27).

Likewise, evidence suggests that LbpA and LbpB form a complex in the outer membrane similar to the TbpAB complex. Two-dimensional gel electrophoresis demonstrated the comigration of LbpA and LbpB, whereas under denaturing conditions, the LbpAB complex is not formed (122). Further, when isolated from an isogenic LbpA mutant, LbpB migrates to a different position under the same native electrophoretic conditions (122).

Structure and functional relationships. The affinity of binding of the wild-type gonococcal receptor to transferrin is comparable to estimates for the mammalian transferrin receptor, with a K_d of 5 to 20 nM (48). The bacterial transferrin receptor, however, is both genetically and antigenically distinct from the mammalian transferrin receptor (44). No studies which precisely measure the affinity or kinetics of binding for lactoferrin with the lactoferrin receptor have been reported. However, competitive blocking experiments with unlabeled lactoferrin ligand and the intact lactoferrin receptor complex indicate that the inhibition of binding occurs at concentrations similar to those used for transferrin in transferrin receptor interaction studies. The binding affinities are therefore in the same range as those of the transferrin receptor, between 10 and 50 nM (A. Schryvers, personal communication).

The specific interactions between TbpA and human transferrin and LbpA and lactoferrin remain to be described. The process must involve the high-affinity binding of transferrin to the receptor complex, the stripping of iron from transferrin, and the transport of iron through the outer membrane into the periplasm. Neither transferrin or lactoferrin is internalized in this process (149).

Data support an interaction between TbpA and the C lobe of transferrin, which is also the domain most highly saturated with iron (5, 177). This interaction is not dependent on the presence of carbohydrate chains on transferrin, indicating that it is a direct protein-protein interaction (108). Recombinant human-bovine transferrin hybrid proteins were designed to determine regions of contact with the transferrin receptor (127). Amino acid residues (aa) 346 to 588, within the carbox-

yl-terminal end of transferrin, were found to be responsible for interacting with the meningococcal receptor; in addition, a region very near the carboxyl terminus has an influence on the avidity of the interaction (127).

Regions of the lactoferrin receptor that interact with lactoferrin have not yet been determined. Attempts to identify regions of lactoferrin that interact with the receptor protein have been made. The N lobe and C lobe fragments of lactoferrin were separated by tryptic digestion, and their binding to the lactoferrin receptor was assessed (174, 176). Both lobes of lactoferrin were bound by the receptor; recent studies have identified two binding regions within the C lobe of human lactoferrin (174). This is in contrast to the case for transferrin, of which only the C lobe interacts with the transferrin receptor. Specific sites of interaction remain to be determined.

Likewise, studies directed at identifying the transferrin-binding domain of TbpA and other important functional domains have begun. Since the crystal structure of TbpA has not been determined, structure-function studies for TbpA have relied on the predicted two-dimensional model. The binding domain for transferrin is most likely located in the extracellular loops of TbpA. Experiments involving the deletion of predicted extracellular loops suggest that L4 and L5 are involved in transferrin binding (29). Consistent with these experiments, overexpressed loop regions, L4 and L5 combined and L5 alone, have been shown to specifically bind transferrin in solid-phase in vitro assays (93). An effort has also been made to demonstrate that L5 and other predicted loops, including L2, L3, L7, and L10, are surface exposed, by using the insertion of detectable epitopes (M. K. Yost and C. N. Cornelissen, presented at the Thirteenth International Pathogenic Neisseria Conference, Olso, Norway, 2002). Additionally, loop deletion analysis has indicated that L8 may be involved in the utilization of iron from transferrin, since loss of this predicted extracellular loop results in a transferrin utilization-deficient but binding-proficient phenotype (29).

The exact roles of TbpB and LbpB in transferrin and lactoferrin utilization by *N. meningitidis* remain unclear. TbpA alone is capable of removing iron from transferrin (61); however, TbpB appears to act as a facilitator in this process (9). Similarly, binding analysis of LbpA and LbpB indicates that both proteins can bind lactoferrin independently, but only an LbpA mutant is incapable of using iron from lactoferrin (115). In nutrition assays, TbpB^{-} meningococci are unable to grow on media supplemented with 1 μM transferrin (74). TbpB-deficient gonococci can grow only poorly under the same conditions (9). TbpB also has the ability to distinguish between apo- and holotransferrin and binds the holotransferrin with a 100-fold-higher affinity, whereas TbpA does not (26, 48, 121, 125, 128, 176). The specificity and high affinity of TbpB for the correct iron-loaded substrate is undoubtedly key to its function in the transferrin receptor complex.

As mentioned previously, TbpB is a bilobed protein and both halves are capable of binding transferrin (28, 126, 166). The interactions of each lobe of TbpB with transferrin appear to be identical, since experiments demonstrate that the same peptide derivatives of transferrin are recognized by both binding domains (129).

Energy from the TonB-ExBD system. Analogous to the TonB-dependent siderophore acquisition systems found in enteric bacteria, *N. meningitidis* requires a *ton* system for utilization of transferrin, lactoferrin, hemoglobin, and haptoglobin-hemoglobin (157). The TonB complex, which consists of TonB, ExbB, and ExbD, is located in the inner membrane and functions to transduce the energy of the proton motive force into conformational changes in TonB-dependent outer membrane transporters (see reference 120 for a review). The *N. meningitidis* TonB, ExbB, and ExbD proteins have roughly 30% amino acid identity with their *Escherichia coli* homologues (157). Reconstitution of the neisserial hemoglobin utilization system in *E. coli* required not only the hemoglobin receptor protein but the neisserial *ton* genes as well (157). Recently, the intracellular replication of *N. meningitidis* within epithelial cells has also been shown to be a TonB-dependent process, implicating TonB in acquisition of intracellular host iron (82). In the infant rat model of *N. meningitidis* septicemic infection, *tonB*, *exbB*, and *exbD* were found to be essential (158).

More is known about the interactions between TonB and the gonococcal transferrin receptor than about the meningococcal transferrin receptor. TonB has been copurified with the gonococcal TbpA protein in the presence and absence of TbpB (77). No chemical cross-linkers were used to artificially maintain this interaction. Furthermore, immunoprecipitation studies allowed the isolation of TbpA-TonB complexes (77). Transferrin was not necessary to detect the interaction, implying that TonB binding occurs in the absence of ligand binding (77). However, the TbpA TonB box, which is a short region of homology near the amino termini of TonB-dependent receptors, was shown to be essential (77). Mutating the TbpA TonB box left the receptor de-energized and insensitive to TonB, similar to the case for TonB box mutations in other TonB-dependent receptors. This TonB box mutant was not affected in transferrin binding, but it did not transport iron and did not efficiently release transferrin from the cell surface. Further, mutations in the TonB box of TbpA were shown to have an effect on the protease susceptibility profile of TbpB (46). This further suggests an interaction between TbpA and TbpB and indicates that this interaction is somehow influenced by the interaction between TbpA and TonB and most likely by the energy state of TbpA (46).

Transport through the periplasm. Several different routes for the translocation of iron across the cytoplasmic membrane are possible in bacteria (see reference 81 for a review). The import of ferric iron acquired from transferrin and lactoferrin into the cytoplasm of *N. meningitidis* requires the Fbp (ferric-binding protein) system (79). Only heme iron can be utilized by *fbp*-deficient meningococci, and not the ferric iron derived from transferrin, lactoferrin, or iron chelates (79). This system is distinct from the periplasmic iron transport systems involved in the transport of iron-siderophore complexes. The Fbp system has low but significant homology to the respective components of ATP-binding cassette (ABC) transporters that are involved in the utilization of sulfate, spermidine, and putrescine (81). Homologous systems include the *hitABC* operon of *Haemophilus influenzae* and the *sfuABC* operon of *Serratia marcescens* (12, 137). ABC transporters couple ATP hydrolysis with substrate translocation across biological membranes. The

Fbp transporter consists of FbpA, a substrate-binding protein; FbpB, a putative inner membrane component of the permease; and FbpC, a putative ATPase (78). An iron-regulated operon, with Fur-binding sites, encodes the genes for the Fbp system (51, 78).

FbpA is one of the major iron-regulated proteins and has been identified in all pathogenic *Neisseria* species (20, 99, 101). It has an apparent molecular mass of 37 kDa and is very basic (pI of >9.35) (97, 98). The role of Fbp in the periplasmic transport of iron was first indicated by its localization to the periplasm and its transient association with ^{55}Fe acquired from ^{55}Fe-labeled human transferrin (19, 40). In vitro studies using purified TbpA, TbpB, and FbpA have shown that FbpA is absolutely unable to acquire transferrin-bound iron unless the metal is freed by the interaction of transferrin with the TbpAB complex (61). No studies describing exactly how FbpA acquires ferric iron from the transferrin receptor or how it relinquishes the iron for transport across the inner membrane have been reported.

The crystal structure of FbpA from *H. influenzae* has been determined, and interestingly, the protein resembles a single lobe of transferrin (35). FbpA and transferrin share some additional characteristics. They both have the ability to reversibly bind ferric iron, and they share similar binding affinities (40). Also, only a single ferric ion binds per lobe (two for transferrin and one for FbpA), and the ferric iron-binding sites are similar (97, 103). The crystal structure revealed that identical amino acid residues in transferrin and FbpA, i.e., two tyrosines, a histidine, and a glutamic acid, coordinate the iron atom but that these amino acids arise from different regions of FbpA than of transferrin (42). Additionally, the FbpA protein completes the octahedral coordination of Fe^{3+} with oxygens from a phosphate and a water molecule, whereas transferrin utilizes the two oxygens from a carbonate molecule (42).

The FbpB protein is a 56-kDa inner-membrane-spanning protein which has some internal homology and a putative interaction domain proposed to bind the 38-kDa FbpC component (81). FbpB may interact with FbpA as well as confer specificity of binding for ferric iron, based on similarity to the inner membrane component of the bacterial permease for maltose, MalF (72, 102). FbpC is expected to provide the energy for transport through the FbpB cytoplasmic permease. FbpC is a putative ATPase and has a "walker box" motif characteristic of the ATPase family of proteins. No detailed molecular studies have been done with the FbpB and FbpC components of the periplasmic transporter system.

Ferric iron in the cytoplasm. Iron serves many essential functions in biological systems. The large majority of bacteria use iron-dependent enzymes in metabolic processes such as DNA replication, electron transfer, and the metabolism of oxygen, peroxide, and superoxide (65). However, in the presence of oxygen, iron can form free radicals which are toxic to the cell. Therefore, sequestration, mobilization, and storage of iron in a nontoxic, bioavailable form are very important to a cell. Little is known about what happens to iron in the cytoplasm. Two types of iron storage proteins have been identified in bacteria, i.e., bacterioferritin (Bfr), which stores both heme iron and nonheme iron, and ferritin, which stores only iron and not heme (11). No iron storage proteins have been described for *N. meningitidis*; however, Bfr has been identified recently in the closely related *N. gonorrhoeae* (41). No homologues of ferritin were found in *N. gonorrhoeae*. Most Bfrs are high-molecular-weight homopolymers, consisting of many subunits; however, the gonococcal Bfr has two similar but nonidentical subunits (41). The genes for the gonococcal Bfr were identified; they are more than likely transcribed as an operon (41). Regulation by the Fur repressor is indicated by the presence of an upstream Fur box sequence; however, recent studies demonstrate that transcription of *bfrA* and *bfrB* increases in the presence of iron, as should be expected for iron storage proteins (64).

The Hemoglobin Receptor HMBR

Every environment within the host, where *N. meningitidis* must survive in order to cause disease, has only trace amounts of heme iron and heme-containing proteins (60, 84, 156). Yet, two independent heme transport systems which recognize heme-containing proteins, hemoglobin (HmbR) and/or haptoglobin-hemoglobin (HpuAB), have been identified in *N. meningitidis* (87, 153). Over half of the clinical meningococcal isolates from serogroups A, B, C, and Y examined expressed both hemoglobin receptors, which suggests an important role for these receptors in causing human disease (130). The first hemoglobin receptor identified in *N. meningitidis* was HmbR (153). The insertional inactivation of *hmbR* prevented the use of hemoglobin, but not transferrin, lactoferrin, or iron-citrate, as an iron source (153). HmbR is responsible for binding hemoglobin with a high affinity, stripping heme from hemoglobin, and transporting the heme into the periplasm. Only heme, and not hemoglobin, is internalized in this process (153). In addition, HmbR preferentially recognizes but is not limited to human hemoglobin, unlike the transferrin and lactoferrin receptors, which are human species specific (155). Furthermore, a mutation in the hemoglobin receptor gene, *hmbR*, had profound effects on the survival of meningococci in an infant rat model of infection, indicating that hemoglobin utilization is important for *N. meningitidis* virulence (153).

Even a small amount of hemolysis would significantly alter the amount of hemoglobin and haptoglobin-hemoglobin available for bacterial growth. There are many possibilities for how meningococci could have access to free hemoglobin: (i) low levels of hemoglobin can be found in normal serum due to spontaneous hemolysis; (ii) a manifestation of meningococcal disease known as disseminated intravascular coagulation, involving the activation of blood clotting mechanisms throughout the body, results in some erythrocyte lysis; and (iii) meningococci, like other bacterial pathogens, may secrete hemolysins and cytolysins. Two proteins belonging to the RTX family of proteins have been identified in *N. meningitidis* (161–163). FrpA and FrpC contain typical RTX domains found in cytotoxins from other gram-negative pathogens. The secretion of FrpA by the heterologous RTX transport system of *E. coli* has demonstrated its relationship to this family of proteins (161, 163). FrpA, however, is rarely found in clinical isolates. In contrast, FrpC has been found in all invasive and most carrier meningococcal strains tested to date (107). Furthermore, high titers of both immunoglobulin G (IgG) and IgA that recognized FrpC were found in the convalescent-phase sera of patients with meningococcal disease (107). If these proteins are

involved in cytotoxicity or hemolysis, the possession of hemoglobin utilization mechanisms by meningococci would be extremely advantageous.

Genetic arrangement and regulation by Fur. The gene encoding the hemoglobin receptor, *hmbR*, is located 178 bp downstream of a gene involved in the catabolism of heme, *hemO* (179). Several potential transcriptional starts and a Fur box sequence can be found in the intergenic region; however, several lines of evidence suggests a transcriptional linkage between the genes. HmbR expression is down-regulated in *hemO* mutants and can be induced in *hemO* conditional mutants (179). The *hemO* gene also has a Fur box regulatory sequence in its promoter region. Fur regulation of *hmbR* was demonstrated by using a Fur titration assay in *E. coli*. The upstream Fur box sequence and intergenic promoter region of *hmb*R, when present in multicopy, were able to titrate Fur away from a Fur-regulated reporter gene (153). In addition, hemoglobin binding by *N. meningitidis* was shown to be iron regulated but not heme regulated (155). The Fur repressor from the closely related *N. gonorrhoeae* has recently been isolated and shown by electrophoretic gel shift assays to bind to the promoter regions of *hmbR* and *hemO* (148).

In contrast with the transferrin receptor of *N. meningitidis*, the expression of HmbR undergoes phase variation due to slip-strand mispairing of poly(G) tracts within the *hmbR* gene (60, 86). The obvious advantage associated with phase-varying surface proteins is evasion of the host immune response. Interestingly, among clinical isolates of serogroups A, B, and C, the phase variation frequency of hemoglobin receptors has been found to vary dramatically, ranging from approximately 10^{-6} to 10^{-2} CFU^{-1}. Frequencies of phase variation were shown to be the genetic traits of particular strains, where two unlinked hemoglobin receptors phase vary with similar frequencies within a given isolate (131). The elevated mutation rates found in a study of epidemic serogroup A isolates were attributed to defects in mismatch repair pathways (131). These studies found a relatively high frequency of hypermutable strains among epidemic isolates, suggesting that the ability to mutate at high frequency may be advantageous to the meningococci (131).

Structure predictions. HmbR is an 89-kDa protein that localizes to the outer membrane of *N. meningitidis*. A signal peptidase I recognition sequence is located in the amino-terminal region, and amino acid sequencing of the mature protein demonstrates cleavage of a 23-amino-acid peptide (111). HmbR shares some weak amino acid identity, between 21 and 22%, with the neisserial transferrin and lactoferrin receptors (153). It also shares homology with heme transporters from other species, including HemR from *Yersinia enterocolitica* (152).

Since the structure of HmbR has not yet been obtained, a two-dimensional model has been developed based on its inclusion in the TonB-dependent family of iron transporters (Fig. 2). The two-dimensional model of HmbR consists of 22 putative transmembrane β-sheets and 11 surface-exposed loops (111). Two putative disulfide bonds are located in L6 and L8. The putative L7 contains an invariable histidine residue and amino acid motifs FRAP (YRVP in HmbR) and NPNL, a highly conserved domain present in all heme-hemoglobin receptors (156). The amino-terminal end of the protein is predicted to extend into the pore of the barrel structure, like the plug domains of the ferric siderophore receptors. Additionally, three-dimensional modeling of HmbR on the structures of ferric siderophore receptors FepA, FhuA, and FecA has also been performed (111). The predicted cork domain of HmbR was superimposed on the cork domain of FecA, and the amino acids which joined the cork region to the beginning of the first transmembrane region corresponded to residues P150 to R152 of HmbR, as indicated in the two-dimensional model. The loop regions of HmbR, L7 and L8, and the intervening transmembrane region corresponded well to the homologous region on FepA. It was also noted that the L7-L8 region of HmbR in the three-dimensional model was marked by a high electrostatic potential, with several glutamic acid residues (E494, E548, and E549) in close proximity to each other.

Structure and functional relationships. The binding kinetics for the interaction between hemoglobin and HmbR have been measured with purified proteins in vitro by using surface plasmon resonance. HmbR has a high binding affinity for hemoglobin, with a K_d of around 13 nM. The association rate, or rate of HmbR-Hb complex formation, is 4.4×10^5 M^{-1} s^{-1}, and the stability, or dissociation rate, of the complex is 6.8×10^{-3} s^{-1} (D. Perkins-Balding, unpublished data).

The region of hemoglobin that directly interacts with HmbR has not yet been identified. However, functionally important domains of HmbR, including a region putatively involved in hemoglobin binding, have been discerned by using a series of HmbR deletions, insertions, and site-directed mutations (111). Mutations exhibiting similar phenotypes in *N. meningitidis* fell into two groups. The first group of mutations affected hemoglobin binding and localized to the middle region of the protein; two putative extracellular loops, L2 (aa 190 to 232) and L3 (aa 254 to 284), marked this putative Hb-binding region. The second group of mutations resulted in a failure to utilize hemoglobin but did not affect proficiency in hemoglobin binding. These mutations localized to a region in the middle to carboxyl-terminal end of HmbR; the putative extracellular loops involved included L6 (aa 420 to 462), L7 (aa 486 to 516), and L10 (aa 661 to 700).

Deletion analysis also revealed that the highly conserved protein motif found in all heme-hemoglobin receptors, within putative extracellular L7 of HmbR, is essential for hemoglobin utilization but is not required for hemoglobin binding. This finding suggests a mechanistic involvement of this motif in heme removal from hemoglobin. In addition, an amino-terminal deletion in the putative cork-like domain of HmbR affected hemoglobin usage but not hemoglobin binding. This result supports a role of the cork domain in utilization steps that are subsequent to hemoglobin binding.

Energy from the TonB-ExbBD system. Hemoglobin utilization mediated by HmbR is a TonB-dependent process. The amino-terminal end of the mature HmbR protein contains a putative six-amino-acid TonB box, a characteristic shared by all TonB-dependent transporters (153). Further, studies have shown that HmbR requires expression of the *N. meningitidis* Ton system to function as a hemoglobin receptor in *E. coli* (157). In addition, a specific interaction with the neisserial TonB protein has been demonstrated in vitro, both by immunoprecipitation analysis and by surface plasmon resonance. The binding kinetics of the HmbR-TonB interaction

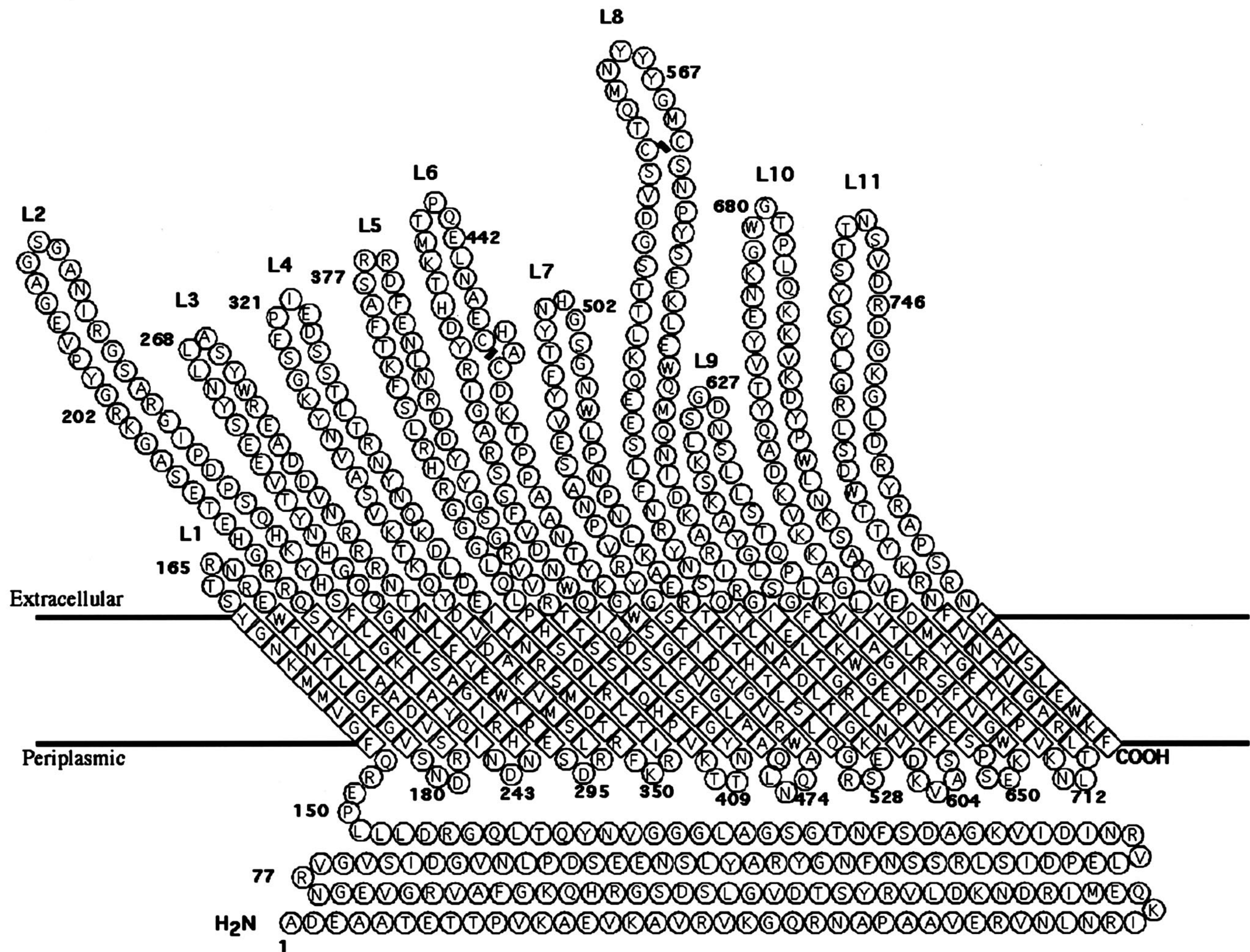

FIG. 2. Topological model of HmbR. The size of each β-sheet was arbitrary taken to be 10 aa residues. L1 through L11 indicate putative surface-exposed loops of HmbR. Numerals indicate amino acid residues of HmbR.

were examined by surface plasmon resonance, and the binding affinity for neisserial TonB is high, with a K_d of 76 nM. The rate of HmbR-TonB complex formation is 1.3×10^5 M^{-1} s^{-1}, and the rate of dissociation of the complex is 9.7×10^{-3} s^{-1} (Perkins-Balding, unpublished data).

Transport through the periplasm. Little is known about the transport of heme from the periplasm through the inner membrane of *N. meningitidis.* The Fbp system is not involved, however, since the use of iron from heme and hemoglobin is unimpaired in an *fbpABC* mutant strain (79). Once HmbR transports heme into the periplasm, it is most likely delivered to the cytoplasm by HemTUV-like proteins. The HemTUV system of *Y. enterocolitica* is a model system for heme-specific ABC transporters in gram-negative bacteria. The transporter consists of a heme-specific periplasmic binding protein, an integral inner membrane permease, and a cytoplasmic ATPase. Even though the complete genome of *N. meningitidis* has been sequenced, proteins homologous with periplasmic heme transporters have not been identified.

Heme in the cytoplasm. Until recently, very little has been known about how microorganisms utilize iron that is sequestered in heme. Some bacteria have been shown to possess enzymes homologous to human heme oxygenase, HO-1. This enzyme is capable of degrading heme to α-biliverdin, with the liberation of free iron and CO (Fig. 3). HO-1 is an inducible isoform of human heme oxygenase which has been found to respond to a diverse group of stimuli. These include heat shock and the presence of heavy metals as well as the substrate and cofactor, heme (114). The response to these stimuli suggests that HO-1 may play a significant role in protection against oxidative stress. Within the last several years, heme oxygenases have been characterized for three human pathogens: *N. meningitidis* (HemO), *Pseudomonas aeruginosa* (PigA), and *Corynebacterium diphtheriae* (HmuO) (124, 138, 179). Thus, it is also plausible that microbial pathogens capable of utilizing heme and hemoglobin as a source of iron may have heme oxygenase-like enzymes for the degradation of heme molecules, not only for the acquisition of iron but perhaps also for heme detoxifi-

cation. *N. meningitidis hemO* mutants were found to be hypersusceptible to heme, supporting this idea (179).

HemO is essential to the utilization of heme and hemoglobin as the sole sources of iron in *N. meningitidis*, as a *hemO*-deficient mutant is unable to utilize either (180). Mechanistically, HemO is identical to *C. diphtheriae* HmuO; both enzymes convert heme to α-biliverdin, with the liberation of iron and carbon monoxide (179). Interestingly, PigA from *P. aeruginosa*, which is 37% identical to HemO, may represent a novel bacterial heme oxygenase. PigA was found to produce a structurally different biliverdin than all previously characterized heme oxygenases (124). Instead of α-biliverdin, PigA yields 70% δ-biliverdin and 30% β-biliverdin.

The crystal structure of HemO from *N. meningitidis* has been determined (Fig. 4) (146). The distal pocket of the heme-HemO complex is significantly smaller, having a solvent-accessible volume of 7.5Å, compared to 53.5Å for the heme–HO-1 complex. The distal helix has a kink of approximately 50° directly over the heme provided by the glycines of the highly conserved sequence ^{116}Gly-Ser-Asn-Leu-Gly-Ala^{121} in HemO, corresponding to ^{139}Gly-Asp-Leu-Ser-Gly-Gly^{144} in HO-1. This kink in the helix is proposed to provide flexibility to the helix, which is required for binding of the substrate (heme) and release of the product (biliverdin). The helix closely approaches the heme, with direct backbone contacts from Gly-139 and Gly-143 in HO-1. The heme propionates and their interaction with the protein are critical in binding and correctly orienting the α-*meso* carbon of the heme for hydroxylation (146). Residues possibly involved in interactions with the heme in HO-1 are Lys-18, Lys-22, Lys-179, and Arg-183, as well as Tyr-134. In the heme-HemO complex, only Lys-16 and Tyr-112, corresponding to Lys-18 and Tyr-134, are absolutely conserved in the structure. Lys-22 is replaced by Thr-20, and Trp-53 largely fills the space occupied by Arg-183 and Lys-179. Mutagenesis studies on Arg-183 of HO-1 have suggested that this residue is involved in orienting the heme within the active site through direct hydrogen bonding to the propionate group. The critical proximal helix histidine residue of HemO (His-23) is absolutely conserved among all heme oxygenases (82).

It remains to be seen whether other bacteria possess heme oxygenases that are not homologous with HmuO, HemO, and PigA or, alternatively, use iron from heme by a different mechanism. Iron acquisition systems are very redundant in bacteria, and this would not be an unexpected solution to the problem of iron limitation.

Haptoglobin-Hemoglobin Receptor HPUAB

The second heme-specific transporter in *N. meningitidis* is HpuAB. HpuAB is a two-component transporter, like the transferrin and lactoferrin receptor proteins of the meningococci. Although it seems that the preferred substrate of the HmbR receptor is human hemoglobin, the HpuAB receptor has a broader spectrum of ligands. HpuAB binds hemoglobin, haptoglobin, and hemoglobin-haptoglobin complexes (87). Studies with non-human-derived hemoglobins have demonstrated no discrimination between different hemoglobins by HpuAB (134). Interestingly, HpuAB also shows enhanced binding of oxidized methemoglobin over reduced hemoglobin (134). Methemoglobin may be an abundant source of iron for meningococci when erythrocytes lyse. Production of methemoglobin is due to dissociation of the tetrameric hemoglobin into hemoglobin α1β1 dimers, the change of ferrous iron to ferric iron in heme, and loss of the liganded oxygen atom (134).

Some meningococci express either HpuAB or HmbR, while others express both (155). Unlike in the meningococci, HpuAB is more than likely the only hemoglobin receptor expressed in the gonococci due to a premature stop codon in *hmbR* (155). The reason that meningococci have redundant hemoglobin receptor proteins is unclear, but it suggests that acquisition of heme, perhaps from various source proteins, is important in pathogenesis.

Genetic arrangement and regulation by Fur. The hemoglobin-haptoglobin receptor HpuAB is encoded on a bicistronic operon. An insertional mutant of *hpuA* was shown to exert a polar effect on downstream *hpuB*, eliminating HpuB expression, and the bicistronic mRNA for *hpuAB* was isolated (89). Primer extension analysis was used to identify the transcriptional start site of the *hpuAB* operon, located 33 nucleotides upstream of the translational start for *hpuA* (89). The *hpuB* gene does not contain its own promoter. The promoter region of *hpuAB* contains a Fur box sequence, and the mRNA transcript was shown to be iron repressible (89). Thus, like for other iron-transport systems of *N. meningitidis*, regulation of the hemoglobin-haptoglobin receptor appears to be under the control of the Fur protein.

Like that of the HmbR receptor and many other virulence-related proteins in *N. meningitidis*, expression of HpuAB varies due to translational frameshifting resulting from slip-strand mispairing of a poly(G) tract within the coding sequence of *hpuA* (88). The rate of phase variation of *hpuAB* is similar to the rate for *hmbR* within any given strain, provided that the number of repeat sequences in the poly(G) tract is equal (131, 132). Interestingly, the frameshifting mutation in *hpuA* correlates not only with a lack of HpuA expression but with a lack of HpuB as well (88).

Structure predictions. The hemoglobin-haptoglobin receptor consists of lipoprotein HpuA and the transmembrane protein HpuB. Labeling of meningococci with [3H]palmitic acid demonstrated that HpuA is acylated (89). This lipoprotein is believed to be anchored to the outer membrane via its lipid moiety and to be largely surface exposed (89). HpuA is 37 kDa, approximately one-half of the size of the lipoprotein components of the transferrin and lactoferrin receptors, TbpB and LbpB. It shares almost no sequence similarity with these proteins (88). In fact, no homologues have been identified for HpuA.

Conversely, HpuB is an 85-kDa outer membrane protein that shares 25% identity and 40% similarity with the TbpA and LbpA components of the ferric iron transporters of meningococci (134). Based on its high degree of similarity to the TonB-dependent receptor family, HpuB is predicted to be a β-barrel structure, forming a pore in the meningococcal outer membrane, which is filled with an amino-terminal 'plug' domain (134). No structural model has been reported for HpuB.

Data suggest that HpuA and HpuB interact, probably forming a receptor complex in the outer membrane. HpuB can be purified by hemoglobin affinity chromatography (39). HpuA, on the other hand, can be isolated only by using hemoglobin

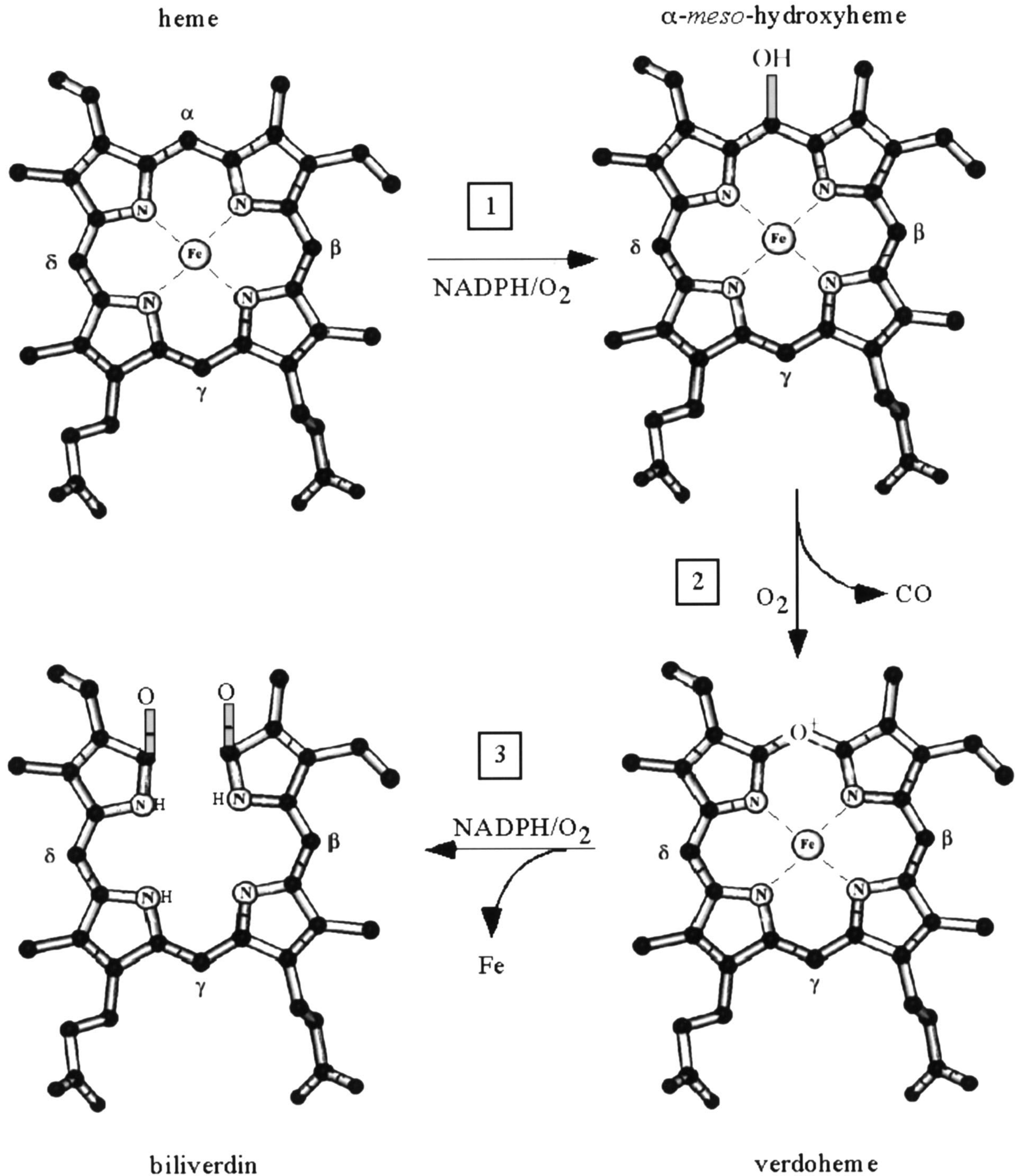

FIG. 3. Chemical steps in heme degradation as defined by studies of eukaryotic heme oxygenases and *C. diphtheriae* HmuO.

agarose in the presence of HpuB, indicating a possible interaction (39). Also, protease susceptibility assays confirmed the surface accessibility of both proteins and indicated a physical interaction between HpuA and HpuB (134). A 21-kDa hydrophilic domain of HpuA, containing numerous protease cleavage sites, was protected from cleavage in the presence, but not absence, of HpuB and remained membrane associated after its proteolytic release from the amino-terminal anchor (134).

Structure and functional relationships. Experimental data suggest that ligand recognition and binding by the two-component HpuAB receptor function differently from those of the two-component transferrin and lactoferrin receptors of *N.*

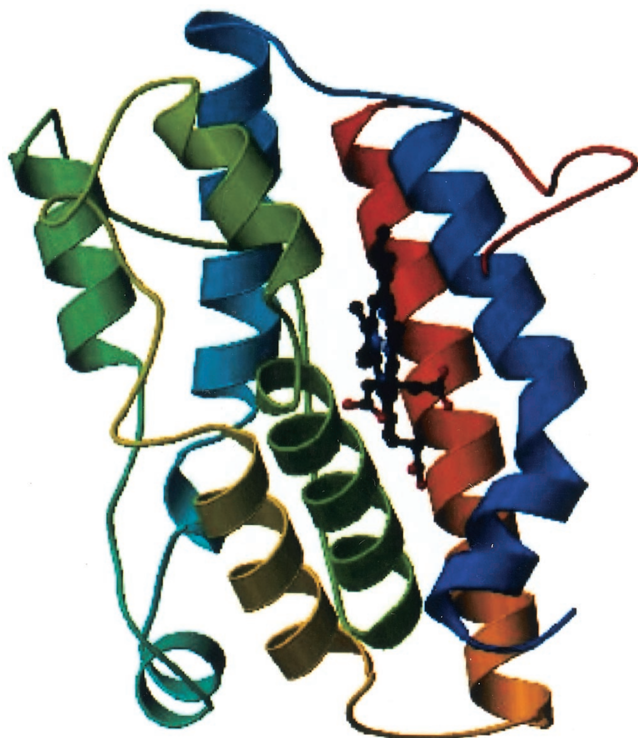

FIG. 4. Crystal structure of *N. meningitidis* heme oxygenase, HemO, with bound heme.

meningitidis. Unlike the accessory function that both TbpB and LbpB serve in transferrin and lactoferrin utilization, HpuA is absolutely essential for meningococcal growth on hemoglobin or hemoglobin-haptoglobin as sole sources of iron (134). Analysis of hemoglobin binding by HpuAB indicates that only one high-affinity binding site exists (134). Conversely, both TbpA and TbpB have been shown to independently bind transferrin with high affinity (48). The affinity of binding of hemoglobin to the single bipartite HpuAB receptor was estimated at 150 nM (134). The single binding site of HpuAB is consistent with the phenotypic observation that mutants deficient in either HpuA or HpuB demonstrate almost no binding to hemoglobin (39, 134). Optimal binding and heme utilization require both proteins. Other receptor functions, however, such as removal of the iron or heme from host carrier proteins, transport through the outer membrane, and interactions with the TonB-ExbBD system, may be similar for HpuAB and the transferrin and lactoferrin receptors.

Energy from the TonB-ExbBD system. Transport of heme iron by HpuAB requires the meningococcal TonB system (88). A TonB box sequence has also been identified in the amino-terminal region of HpuB (89). Recent studies which investigated the roles of TonB and the proton motive force in the binding of hemoglobin to HpuAB and in the conformation of HpuAB in the outer membrane have been done (134). The absence of TonB or a proton motive force had an influence on the protease susceptibility of HpuB, suggesting that TonB causes a conformational change in HpuB. Additionally, the presence of TonB and the proton motive force was not absolutely required for hemoglobin binding, but binding was decreased by 10-fold in a de-energized cell. The binding affinity had not decreased; therefore, a decrease in the number of binding-competent receptors was indicated. In this system, it was proposed that TonB serves to enhance a productive interaction between HpuA and HpuB, which would yield more two-component, single-binding-site receptors, in a binding-competent state at the cell surface (134).

Transport through the periplasm. Heme transported through the HpuAB receptor into the periplasm is expected to use the same system for delivery of heme into the cytoplasm as heme transported through HmbR (see above).

Other Potential Iron Transporters

Seven potential genes with products having homology to TonB-dependent transporters have been identified from the *Neisseria* genome sequencing projects (164). Two out of three genes which have been examined are present in the *N. meningitidis* DNA sequence; these are *tdfF* and *tdfH* (164). Only TdfH appears to be expressed as determined by Western blot analysis (164). Based on homology to the FpvA pyoverdin transporter of *P. aeruginosa* and the FhuE coprogen transporter of *E. coli*, TdfF is predicted to be a siderophore transporter, and TdfH exhibits low homology to the HasR hemophore receptor of *S. marcescens* (164). In addition, two outer membrane proteins, which bind heme, have been identified in *N. meningitidis*; these remain uncharacterized (83).

Vaccine Potential and Trials

There are various reasons to consider the transferrin receptor of *N. meningitidis* a good vaccine candidate. Two of the most obvious are localization to the outer membrane, where it is surface accessible, and the lack of phase variation. In addition, the transferrin receptor has been identified on every clinical meningococcal isolate studied to date (47). There is also good evidence that the transferrin receptor is expressed in vivo during infection. *N. meningitidis* taken directly from cerebrospinal fluid of a patient with meningitis demonstrated binding to gold-labeled human transferrin and was expressing TbpB (3). Meningococcal infection in mice can be enhanced by adding iron-saturated human, but not bovine, transferrin, indicating that the receptor-mediated uptake of iron is working in vivo and transferrin is effective in supplying the required iron in this environment (69, 140). In addition, antisera from patients recovering from meningitis contain cross-reactive antibodies to TbpA and -B (4, 62). Further, asymptomatic carriers of *N. meningitidis* have high antibody titers to the Tbps, whereas healthy adults have almost none (62).

More attention has been given to TbpB as a vaccine candidate than to TbpA. Antibody recognition to TbpA is conformation dependent, and antibodies to the protein are not bactericidal (18, 50). However, recently TbpA was shown to protect against meningococcal infection in a mouse model when administered as the sole immunogen (169). The advantage of using TbpA over TbpB is that it is less variable, and it may be protective against a wider range of meningococcal strains (169).

TbpB has been shown to be protective in animal models after both active and passive immunization (1, 50, 92). Antibodies to TbpB, unlike those to TbpA, elicit strong complement-mediated bactericidal activity, and humans mount bactericidal antibody responses to TbpB upon infection (2, 4, 92, 118, 119). Again the disadvantage of using TbpB as an immunogen is its heterogeneity, but there is evidence for conserved epitopes among different TbpBs (173). At least two TbpB representatives will be required in a vaccine for broad-spectrum coverage (135, 145). Recently, a mucosal delivery system was examined for efficacy with TbpB (43). The highly efficient secretion mechanism for filamentous hemagglutinin from *Bordetella pertussis* was used to secrete a recombinant TbpB; the recombinant TbpB was created as a fusion to the amino-terminal end of filamentous hemagglutinin, which contains essential secretion signal sequences (43). The recombinant *B. pertussis* was then used to deliver TbpB intranasally in mice. Antigen-specific antibodies, IgG and IgA, which had complement-mediated bactericidal activity against *N. meningitidis* were detected (43).

Whether the meningococcal lactoferrin receptor proteins can elicit a protective immune response in the host needs to be evaluated. The lactoferrin receptor has been considered a promising vaccine candidate for many of the same reasons as the transferrin receptor; it is surface accessible and is expressed on all meningococcal strains examined to date. However, unlike the transferrin receptor, the *lbpAB* operon may experience phase variation (151). The LbpA portion of the lactoferrin receptor appears to be antigenically stable, based on the cross-reactivity to monoclonal antibodies with 70 different meningococcal strains (112). The human antibody response to LbpA has been analyzed by using two LbpA proteins from different *N. meningitidis* strains and 70 samples of convalescent-phase sera from patients with meningococcal disease (75). The findings indicated that fewer than 50% of sera contained IgG that recognized LbpA, and the response was not uniform between the two different LbpA proteins. Hence, LbpA, like its homologue TbpA, does not elicit a highly cross-reactive antibody response.

The human immune response to meningococcal LbpB has not been reported, but data from LbpB of *M. catarrhalis* indicate that patient sera elicit a strong immune response to the LbpB of this pathogen (25, 139). Further, the TbpB homologue of LbpB is reported to elicit a strong bactericidal antibody response in humans. Unfortunately, TbpB has been found to have a high degree of sequence variability (47 to 82% identity) between strains. When the sequences of LbpB in five serogroup B strains were compared, there was 70 to 80% amino acid identity, which is a considerable degree of variability and is disadvantageous for vaccine development (116).

The vaccine potentials of HmbR and HpuAB have not been explored, in part because there are no obvious advantages in using the hemoglobin receptors over other iron transport proteins, specifically the transferrin receptor. The benefits of using HmbR are much the same as with the transferrin receptor, including its surface accessibility and its presence on all invasive strains tested to date. Analysis of a limited number of convalescent-phase serum samples demonstrated reactivity to HmbR, suggesting its expression in vivo (I. Stojiljkovic, unpublished data). Although the expression of HmbR in vivo has not been unambiguously confirmed, the expression of other hemoglobin receptors in vivo, such as those of certain *Haemophilus* species, has been reported. The in vivo expression of the outer membrane hemoglobin receptors in *H. influenzae* has been demonstrated in the human model for acute otitis media, and the hemoglobin receptor for *Haemophilus ducreyi* has been shown to be essential for infection in a human model system (6, 172). A strong disadvantage associated with both HmbR and HpuAB as vaccine candidates is the phase variation of their expression and sometimes a very high frequency of switching to "off." The best fit for these receptors with respect to vaccine development would be as a second immunogen, perhaps enhancing the efficacy of a vaccine that confers less than ideal protection.

Studies of hemoglobin receptors in *N. meningitidis* may not ultimately lead to a protective vaccine against the pathogenic meningococci; however, knowledge of these systems has led to the development of bactericidal compounds which use heme transporters for delivery into the cell (30, 154). Heme transporters are not unique to the meningococci. Many other pathogenic bacteria have heme iron acquisition systems, and others almost certainly remain to be described (84). The development of broad-spectrum antibiotics that use a common portal of entry, such as heme transport systems, warrants attention in a time when antibiotic resistance is ever increasing. Noniron metalloporphyrins, such as gallium protoporphyrin IX, have demonstrated strong and broad antibacterial activity and are currently under investigation (154).

CONCLUDING REMARKS

Four identified iron acquisition systems, the ability to use multiple sources of iron for survival, the redundancy and phase variation of heme iron transport systems, and the regulation of known virulence factors by iron attest to the importance of iron for *N. meningitidis*. It has been further demonstrated that the pathogenesis of meningococci is negatively affected by the absence of the transferrin and hemoglobin receptor proteins in vivo. The essential requirement for iron and its role in pathogenesis, however, are not unique for *N. meningitidis*; many other bacterial pathogens have demonstrated an essential requirement for iron. There are several aspects of meningococcal iron acquisition that are different from most other bacterial systems, as well as some common themes shared with other bacteria.

Some of the uncommon features of meningococcal iron transport systems include two-component receptors, iron removal from host proteins, a lack of siderophores, the phase variation of heme transport systems, and the existence of heme oxygenase. (i) *N. meningitidis* and other closely related bacteria (such as other *Neisseria* species and *Pasteurella* species) are the only species to express two-component outer membrane receptors. Most other bacteria have single-unit transporters in addition to secreted molecules, such as siderophores or hemophores (42). This is not a uniform feature, however, since the meningococcal hemoglobin receptor, HmbR, consists of a single protein unit. (ii) Also unusual, but not unique, is the lack of siderophore production by meningococci accompanied by the potential use of siderophores secreted by other microorganisms (38). Although the search for a meningococcal sid-

erophore has spanned many years, the possibility remains that one exists but has not yet been identified. (iii) The iron transporters of meningococci are different from other transporters because they must remove iron or heme from high-affinity associations with host carrier proteins, in addition to their transport functions. As mentioned previously, the interactions between host carrier proteins and iron are extremely strong, and it remains unclear how meningococcal receptors are able to destabilize or disrupt these interactions. (iv) Like many other genes of the neisseriae, the hemoglobin and haptoglobin-hemoglobin receptors are subject to phase variation; this is not typical of other bacterial iron transport systems. (v) Finally, the presence of a heme oxygenase in meningococci, which serves to catabolize and detoxify heme, is currently an uncommon feature of bacteria. Many bacteria are expected to possess unidentified heme oxygenases, and two others, including one from a gram-positive bacterium, have been identified already.

Some of the commonalities, and primary features, between iron transport in *N. meningitidis* and other bacterial iron transport systems include regulation by iron via the Fur repressor protein, transport through the periplasm by ABC-like transporters, membership in the TonB-dependent family of receptor proteins, and TonB-dependent transport functions. (i) Thus far, all of the described iron transporters in *N. meningitidis* appear to be iron regulated through the Fur repressor protein. The Fur regulon in *N. meningitidis* may in fact consist of a broad array of genes, since genes involved in major catabolic, secretory, and recombination pathways of the closely related *N. gonorrhoeae* have been identified as part of the Fur regulon (148). (ii) Ferric iron transport, and possibly heme iron transport, requires an ABC transport system, consisting of a periplasmic binding protein, transmembrane permease, and inner-membrane-associated ATPase (FbpABC) (see above). Ferric siderophore transporters and related transporters use similar ABC transport proteins, of which the FhuBCD system is the best characterized. (iii) Limited, but significant, homology to the ferric siderophore receptors places a component of each meningococcal receptor, namely, TbpA, LbpA, HmbR, and HpuB, in this family of proteins. Three members of this family, i.e., FepA, FhuA, and FecA, have been crystallized. Structures reveal monomeric transmembrane β-barrels that form pores, which are plugged from the periplasm by a globular amino-terminal domain. No crystal structure data are available for the meningococcal iron transporters; therefore, predictions of structure are made based on their inclusion in this protein family. (iv) All of the iron transporters in *N. meningitidis* have shown dependence on the energy-transducing Ton system. This is characteristic of all members of this protein family. Important questions remain unanswered for all TonB-dependent iron transport systems, not only those found in *N. meningitidis*. How does the Ton system transduce energy from the proton gradient of the cytoplasmic membrane to the outer membrane, and how do outer membrane transporters receive and respond to this energized signal?

Despite the large volume of information known about iron transport in *N. meningitidis*, our understanding remains shallow. So many questions remain unanswered. Some of these are as follows. (i) What are the structures of these different transporters? (ii) What is the mechanism of iron stripping and transport? (iii) How is TonB involved? (iv) How is the cell protected from the toxicity of free iron and free heme? (v) Why are there so many different iron transport systems in meningococci? (vi) Why are there seemingly redundant systems? (vii) Why is there phase variation of the hemoglobin and haptoglobin-hemoglobin receptors? (viii) What is the mechanism of TonB-independent heme uptake reported for meningococci, and does there exist a second set of TonB-ExbBD proteins, as identified in species such as *V. cholerae*, *P. aeruginosa*, and *S. marcescens*, which functions in this process (52, 105, 109, 157, 178)? Answers to these questions will enhance our understanding of iron transport and utilization by *N. meningitidis* and perhaps lead to the development of an effective vaccine.

ACKNOWLEDGMENTS

We thank William Shafer and Gordon Churchward for their support. We also thank Andy Rasmussen for useful discussions of the manuscript.

This work was supported by Public Health Service grants AI472870 and AI045883.

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