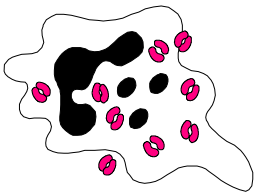
Proteins



Environmentally
Regulated

Neisserial iron regulated stress proteins: What do they do? What can we do with them?

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A decade of work by many able investigators has identified many proteins in the pathogenic Neisseria that are produced in increased amounts during various types of stress, including iron deprivation and oxygen deprivation. This minireview will summarize briefly what is known about iron-regulated proteins, and their roles in pathogenesis and vaccine development. Space limitations prevent review of work on the anaerobically expressed proteins (Ani) (1, 2).

Iron regulated proteins. After one to two mass doublings in vitro under conditions of reduced or absent iron availability, *Neisseria gonorrhoeae* (Ng) and *Neisseria meningitidis* (Nm) produce a variety of iron repressible proteins (Frps or Irps) (3). Many of these are on the cell surface, either in the outer membrane or in the periplasm, and they have been studied intensively. Others are probably in the cytoplasm. By means of two dimensional gel electrophoresis, a surprisingly large set of Ng Frps recently was identified, the genes for which apparently are under transcriptional control of the iron-dependent regulatory protein Fur (4). Two dimensional gels also identified a relatively large set of Ng proteins that are expressed only during exposure to high concentrations of iron (iron inducible proteins, or Fips) (4). Some of the Fips also are located in the outer membrane (S. Carson, C. Elkins personal communication), and may possibly be involved either in protection against iron-related oxygen derived toxic radicals or in cell adherence; the latter inference is based on studies that demonstrate increased Ng adherence in the presence of high iron concentrations (5). Virtually nothing is known about the structure, function and regulation of Fips.

Iron transport: two component receptors. Many of the Frps are involved in uptake of essential iron from the environment. Since iron can be toxic, it is not surprising that systems evolved that regulate iron uptake, depending on the intracellular iron concentration. We now understand that both Ng and Nm produce specific receptors for binding different iron ligands, and that these receptors apparently are built on a similar model. Each appears to be a two component system, with a pair of genes under control of a single Fur-regulated promoter. This concept is well established for the transferrin (Tf) receptor, and is suggested by still incomplete data for each of the lactoferrin (Lf) and hemoglobin (Hgb) receptors. The first gene in the operon apparently encodes a lipoprotein, and the second an integral outer membrane protein that belongs to the family of TonB-dependent receptors. This has been extensively studied in the case of the Ng and Nm Tf receptors, which are very closely related in every detail (6). Each receptor is made up of two Tf binding proteins (Tbp). The lipoprotein Tbp2 is the product of the Fur-regulated gene *tbpB*, (7, 8) which is located immediately upstream of the gene *tbpA* for the integral outer membrane protein Tbp1 (8, 9). The Nm and Ng Lf receptors also appear to be made up of a lipoprotein (Lbp2) and a TonB dependent integral outer membrane protein (Lbp1) (10 and G. Biswas, unpublished data). The Nm and Ng hemoglobin (Hgb) receptor designated

Hpu also may be similarly designed, with both a lipoprotein (L. Lewis and D. Dyer, personal communication) and a TonB dependent integral outer membrane protein working in concert to functionally bind Hgb and/or HgB plus haptoglobin.

The Tf receptor. Tbp1 is essential to entry of Fe from Tf, whereas Tbp2 facilitates Tf binding but is not essential for Fe entry (7). Tbp2 binds iron-loaded Tf preferentially, whereas Tbp1 does not discriminate between iron-loaded or iron-free Tf (11). Both Tbp2 and Tbp1 are surface-exposed, and Tbp2 may be only loosely tethered to the outer membrane, perhaps by its lipid moiety, since it is easily released from whole cells or membranes by certain detergents (11). Formal proof has not been obtained for either physical proximity of Tbp1 and 2 or for protein-protein interactions between these proteins in the membrane, but a variety of evidence suggests that they occur together in patches on the membrane, with more copies of Tbp2 than Tbp1 (11). Recent evidence shows that the Tf receptor exists in more than a single conformational state (12), depending on whether it is effectively energized by the newly-discovered Ng TonB ExbBExbD system (13). A model has been constructed which suggests that Tbp2 helps to promote binding of FeTf to the receptor whereas Tbp1 may serve as a gated TonB dependent channel for entry of Fe released from Tf (6, 11), analogous to the now well-documented TonB-dependent gated porin activities of the *E. coli* iron-siderophore receptors FepA (14) and FhuA (15).

Function of the Tf receptor in vivo. The role of the Ng Tf receptor in pathogenesis of human mucosal infection has been studied very recently in male volunteers in Chapel Hill (16). The question addressed was whether expression of the Ng Tf receptor was necessary for establishing urethral infection in the volunteers. To answer this question, a *tbpB tbpA* deletion mutation was constructed in FA1090, which is a natural Lf^- (lactoferrin receptor deficient) strain. Thus the challenge strain for the experiments was unable to express either a functional Tf or Lf receptor, although it was wild-type in every other parameter. Results showed that 0 of 5 volunteers inoculated with over 10^6 cfu of the Tf receptor mutant strain (Tf^-Lf^-) developed urethral infection, as compared to 3 of 3 volunteers inoculated with a similar dose of the isogenic Tf^+Lf^- strain, and over 90% of historical controls inoculated with the same Tf^+Lf^- FA 1090 strain in previous experiments (12). Thus, under these experimental conditions the Tf receptor appeared to be essential for successful colonization of the normal male urethra, which is surprising in some respects because mucosal surfaces are rich in Lf but normally have very little Tf or other serum proteins. These experiments do not disprove the notion that expression of the Lf receptor would assist in establishment of mucosal infection; it is entirely possible that challenge with a Lf^+ derivative of FA1090 would result in an ID80 of less than the $1x10^6$ cfu dose calculated for the Tf^+Lf^- FA1090 used for all human challenge in Chapel Hill to date (16). It also is conceivable that experimental urethral inoculation of male volunteers with a small catheter results in sufficient transient trauma to allow serum proteins such as Tf to exude onto the urethral mucosa, which could result in an apparent dependence on the Tf receptor for optimal infection of the volunteer. Despite these qualifications, these results are exciting because they strongly suggest that the Ng Tf receptor is very important to mucosal infection, in addition to its presumed essential role in systemic (blood, joint, central nervous system) sites. Presumably, the same conclusion is pertinent to meningococci, and to other mucosal pathogens such as *Hemophilus influenzae* that make similar Tf receptors.

The Lf receptor. All Nm and about 50% of clinical isolates of Ng produce a functional Lf receptor (17). The Lf receptor in both Nm and Ng contains a TonB-dependent integral outer membrane protein designated Lbp1, which is quite similar in predicted amino acid sequence to Tbp1 (10, 18). In addition, limited DNA sequencing upstream of *lbpA* (the structural gene for Lbp1) shows a gene in both Nm and Ng that is very similar to *tbpB*, tentatively identified as *lbpB* (10, and unpublished data of G. Biswas). Transposon insertions into the upstream gene in Ng abolish Lf receptor binding activity (G. Biswas, unpublished data). This suggests that Nm and Ng also produce a Tbp2-like protein, although it has not yet been identified by biochemical techniques, and the entire DNA sequence of *lbpB* has yet to be determined in either Nm or Ng.

The functional importance of the Lf receptor is unclear. Without doubt, mucosal colonization and infection does not absolutely depend on a Lf receptor because many nonpathogenic Neisseria successfully colonize the nasopharynx despite being unable to use Lf as a sole iron source in vitro (17). Moreover, all tested *H. influenzae* (19) and 50% of Ng are unable to utilize Lf as a sole iron source, (17), and experimental male urethral infection with the Lf^- Ng strain FA1090 is successful. Little else is known about the Nm or Ng Lf receptor except it is functionally dependent on the TonBExBExD system in Ng (13) and the molecular basis for failure of many Ng strains to produce a functional Lf receptor is mutation either in *lbpA* or in a region immediately upstream of *lbpA* (18). Presumably a functional Lf receptor assists but is not essential to mucosal infection by helping to scavenge iron from Lf, although alternative functions such as binding of otherwise bactericidal peptides derived from Lf have not been experimentally examined yet.

The Hgb receptors. Both Nm and Ng produce iron-repressed receptors that bind Hgb, or Hgb plus its binding protein haptoglobin (20, 21), and which are essential for iron uptake and growth from Hgb under conditions where HgB is the sole available iron source. Nm produce two similarly sized (ca 76kDa) but genetically and structurally unrelated receptors with apparently similar function, now designated either HpuB (20, and L. Lewis and D. Dyer, personal communication) or HmbR (21) respectively. Ng apparently possesses genes for both of these Hgb receptors, but only HpuB are expressed and functional in vitro (22). The Ng *hmbR* gene is mutated in strain MS11, apparently explaining its lack of expression in gonococci (J. Stojiljkovic, personal communication to C.E. Elkins). Recently, Lewis and Dyer showed that there are two adjacent genes in an *hpu* operon, designated *hpuA* and *hpuB*. The *hpuA* DNA sequence predicts that HpuA is a lipoprotein, whereas *hpuB* encodes a TonB-dependent family member of integral outer membrane protein (20, and L. Lewis and D. Dyer, personal communication). Mutations in the Ng *tonB*, *exbB*, or *exbD* genes abolishes function of the Ng HpuB receptor (13), analogous to the effects of a specific *hpuB* mutation (22). Interestingly, the Ng HpuB receptor undergoes a high-frequency in vitro apparent phase variation from "off" to "on" when grown on media with Hgb as a sole iron source (22), possibly due to slipped-strand mispairing in a polypyrimidine tract observed in the upstream *hpuA* gene (L. Lewis and D. Dyer, personal communication). There are no obvious explanations for the advantage of having two Hgb receptors (HpuB and HmbR) in Nm. Phase variation of a single expressed gonococcal HpuB receptor could be a selective advantage in women during menses.

Other iron uptake systems. Both Nm and Ng can utilize heme, citrate, aerobactin and possibly enterochelin as iron sources in vitro (23). Less is known about the mechanism or physiological

significance of iron uptake from these sources. Mutations in Ng *tonB, exbB,* or *exbD* do not reduce iron uptake from heme or citrate (13), suggesting that if there are specific receptors for these systems, they are not in the TonB-dependent family of receptors.

Much work has focused on another major iron repressed outer membrane protein found in all strains of both Nm and Ng designated FrpB, whose predicted protein sequence shows it is a member of the TonB-dependent family of outer membrane proteins (23, 24). Nevertheless, careful experiments failed to prove that FrpB plays a specific role in iron uptake from any presently studied iron source (23). DNA sequencing in Ng downstream from *frpB* (25) identified three open reading frames that encode proteins that are highly related to the periplasmic and cytoplasmic proteins involved in uptake of phenolate-siderophores in *Vibrio anguillarum* and *Campylobacter coli*, implying a possible role for such a system in Ng.

Other iron-repressed proteins. In addition to the outer membrane receptors for various specific iron sources, both Nm and Ng produce a periplasmic iron-transport protein designated Fbp1 which binds Fe^{3+} from any source after it gains passage through the outer membrane (26, 27). Transport through the cytoplasmic membrane apparently is mediated by the products of two linked genes, *fbpB* and *fbpC* (28).

Vaccine implications. Considerable attention is being given to potential new vaccines for Nm based on the Tf receptor. Bactericidal and mouse-protective anti-meningococcal Tbp2 antibodies have been demonstrated, and they exhibit considerable cross-reactivity against various Nm strains despite extensive variability in amino acid sequence of Nm Tbp2 proteins (29-32). Antibodies are produced to these proteins during natural infection (33). There is somewhat less variability of Ng Tbp2 proteins (34), suggesting that Ng Tbp2 is also an attractive potential vaccine target. There is convincing evidence for surface-exposure of Ng Tbp1 including protease accessibility, Tf binding to whole cells, and ability of certain polyclonal anti Tbp1 sera to bind whole gonococci (34). Since Tbp1 is much less variable than Tbp2 in both Nm and Ng (6, 34), Tbp1 deserves further study as a vaccine target. Another iron-repressible protein with vaccine potential is FrpB, since it is the target for Nm strain-specific bactericidal monoclonal antibodies (35-36) and is relatively antigenically conserved in both Nm and Ng (J.T. Poolman personal communication, unpublished data of M Beucher, J Fu and PF Sparling). Antibodies are also produced against FrpB in natural infection (37).

Concluding remarks. Much has been learned about stress proteins in the pathogenic neisseria, but at least as many questions remain unanswered. Some of these proteins are certainly important in pathogenesis (even essential), and some may be vaccine targets. There is much to be learned about all of the actual or putative iron uptake systems, including why so many are needed, and in what circumstances they are functional.

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Function and virulence studies of the gonococcal transferrin receptor

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The gonococcal transferrin receptor consists of two iron-regulated proteins, Tbp1 and Tbp2. Tbp1 shares homology with TonB-dependent, integral, outer membrane receptors, is necessary for iron-uptake from transferrin (Tf) (1) but is not capable of discriminating between apo and ferrated forms of Tf (3). Tbp2 is lipid-modified, is not essential for iron-uptake from Tf (2), and binds ferrated Tf preferentially (3). We have proposed a model of Tf-iron utilization in which Tbp1 serves as the integral outer membrane pore through which iron traverses the outer membrane after it is removed from transferrin by an unknown mechanism (4). Tbp2 may serve to increase the specificity of the receptor for ferrated Tf and be important in release of apo Tf from the high affinity receptor after iron has been removed (4).

We have evaluated physical association and function of Tbp1 and Tbp2 using Tf-binding and protease-accessibility assays. The gonococcal Tf receptor has a high affinity for Tf with a Kd in the range of 1-16 nM. The Tf-binding parameters of Tbp1 and Tbp2 in isogenic mutants are distinct from those of the wild-type parent, which expresses at least two conformational states of the transferrin receptor (3). Similarly, protease-accessibility experiments indicate that Tbp2 is in a trypsin-sensitive state in a Tbp1$^-$ mutant, while Tbp2 is partially protected from trypsin in the wild-type strain. We have developed a model of the transferrin receptor in which the two Tf-binding proteins in the wild-type strain functionally interact to create a receptor that is capable of assuming at least two conformations. One conformation has a high affinity for transferrin (Kd = 1nM) and Tbp2 is in an "open", protease-sensitive state. The other conformation has a lower affinity for transferrin (Kd = 16 nM) and Tbp2 exists in a "closed", protease-resistant state. We created a Tbp1 mutant analogous to "TonB-box" mutants described in *E. coli* (5). This mutant, in which Ile16 of Tbp1 is changed to Pro16, is unable to grow on or internalize iron from Tf while it remains Tf-binding competent. Analysis of this and other deenergized mutants (6; and Biswas, Anderson, and Sparling, in preparation) suggests that interconversion between the two Tf receptor conformations is dependent upon the energy status of the cell.

To test the virulence contribution of the Tf receptor, we created a *tbpA/B* deletion mutant of gonococcal strain FA1090 that cannot utilize Tf-bound iron and determined its infectivity in a human challenge model of gonococcal urethritis (7). Strain FA1090 is natively unable to utilize lactoferrin (LF)-bound iron and does not express the phase-variable hemoglobin receptor. None of five volunteers inoculated with 1-2 x 10^5 CFU of the Tf$^-$ mutant exhibited signs of urethritis during the course of the four to six day challenge. In contrast, 1 x 10^5 CFU of wild-type FA1090 causes urethritis in 90-100% of subjects. Some volunteers inoculated with the mutant excreted viable gonococci in their urine; however, compared to wild-type controls, the number of gonococci recovered was decreased 2-3 logs. Of the few gonococcal survivors that were recovered from the urine, all retained the phenotypes of the original inoculated mutant. We

conclude that in the absence of functional lactoferrin and hemoglobin receptors, the Tf-receptor deficient gonococcal mutant is greatly attenuated for initiation of urethritis and for survival in the male urethra.

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Periplasm-to-cytosol free iron transport by pathogenic *Neisseria*

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High affinity iron acquisition by pathogenic *Neisseria* has become a paradigm for other bacterial pathogens that specifically sequester host transferrin or lactoferrin to their cell surfaces by way of receptors (1). Subsequent to this binding event is the removal of ferric iron, Fe(III), from the host protein and its transport across the outer membrane in an energy-dependent manner (2). The energy required for the removal and transport of Fe(III) is presumably mediated by a TonB-dependent process, however the precise mechanism for this process has not been elucidated. What is clear is that iron removed from transferrin or lactoferrin is reversibly deposited on the periplasmic Fe(III)-binding protein referred to as FbpA (2). This initiates the process of periplasm-to-cytosol Fe(III) transport by way of a classic active transport process.

Biochemical, crystallographic, and site-directed mutagenesis studies from our laboratory have demonstrated that FbpA is a member of the classic periplasmic binding protein family (3). FbpA is a novel member of this group in that it is the only known Fe(III)-binding protein. The property of Fe(III) binding and its periplasmic binding characteristics led us to hypothesize that FbpA is a functional homologue of a half-transferrin. The range of metal ions bound by Fbp and transferrin have been previously reported and include gallium, Ga(III), and terbium, Tb(III). We have prepared site-directed mutations in suspected residues involved in Fe(III) binding by FbpA. This analysis has demonstrated three classes of mutations: (i) those that do not affect metal binding, (ii) those that significantly decrease metal binding, and (iii) those that abrogate metal binding. The remarkable similarity of the contingent of amino acids utilized for metal coordination by FbpA and transferrin demonstrates the functional homology of these proteins.

The genetic organization of FbpA as the protein product of the first gene, *fbp*A, in a three gene operon has been recently reported (4). In addition two linked genes, *fbp*B and fbpC, are proposed to make up a classic ABC transporter that is critical to the periplasm-to-cytosol transport of Fe(III) across the cytoplasmic membrane. Analysis of the predicted FbpB amino acid sequence demonstrates hydrophobic and putative membrane-spanning regions characteristic of a cytoplasmic permease. A similar analysis of FbpC reveals classic Walker motifs that are associated with nucleotide-binding proteins. However, biochemical analysis of FbpC demonstrated additional characteristics of membrane association and substrate specificity that were not predicted based on homology to described nucleotide-binding proteins that operate in active transport processes. Both the cytoplasmic permease and nucleotide-binding properties are required for classic ABC transporter activity (4, 5).

The extrapolation of the preceding genetic predictions have been investigated by establishing a functional model for studying iron transport by FbpABC in an *E. coli* background (4, 5). These results have demonstrated the absolute requirement for all three components of the FbpABC

operon as well as the toxicity associated with the FbpB component. Furthermore, single amino acid mutations that decrease the affinity of FbpA for metal ions by only one order of magnitude can result in rescue of the organism from the Fbp-mediated toxicity to Ga(III)

These studies describe the biochemical basis for the periplasm-to-cytosol transport of Fe(III) characteristic of pathogenic *Neisseria*. Furthermore, they underscore the importance of the FbpABC operon as an important contributor to success of these pathogens. In a larger context, these studies provide further evidence for the importance of pathogenic *Neisseria* multiplication within the host environment as a critical component associated with disease outcome.

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The *N. meningitidis* hemoglobin receptor genes and interaction between the hemoglobin receptor and the hemoglobin molecule

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We have recently cloned and characterized the hemoglobin receptor gene, *hmbR*, from *N. meningitidis* serogroup C isolate (1). The *N. meningitidis hmbR* mutant was unable to use hemoglobin as an iron source but was still proficient in heme utilization (1).The hemoglobin utilization-deficient mutant of *N. meningitidis* was attenuated in the infant rat model of meningococcemia, underling the importance of hemoglobin as an iron source for meningococci (1). Both *N. meningitidis* and *E. coli* cells expressing HmbR protein were able to bind biotinylated hemoglobin and the binding was specifically inhibited by unlabeled hemoglobin but not heme (2, 3). The HmbR-mediated Hb binding activity of *N. meningitidis* cells was shown to be iron-regulated. Presence of hemoglobin but not heme in the growth media stimulated HmbR-mediated hemoglobin binding activity. The efficiency of utilization of different hemoglobins by HmbR-expressing *N. meningitidis* cells was shown to be species specific; human hemoglobin was the best source of iron followed by horse, rat, turkey, dog, mouse and sheep hemoglobins. N,N'-methylated hemoglobin was the worst source of iron for *N. meningitidis* cells (2). These results indicate that the HmbR receptor is able to recognize globin part of hemoglobin molecule. We have cloned and characterized *N. meningitidis* serogroups A, B, and *N. gonorrhoeae MS11 hmbR* homologues (1, 2). The deduced amino acid sequences of these Neisserial receptors were highly related with an overall 84.7% identical amino acid residues (2). The phenotypic characterization of HmbR mutants in some clinical strains of *N. meningitidis* suggested the existence of two unrelated hemoglobin receptors. The HmbR-unrelated hemoglobin receptor was shown to be identical to Hpu, the hemoglobin-haptoglobin receptor of *N. meningitidis* (2, 4). The Hpu-dependent hemoglobin utilization system was not able to distinguish between different sources of hemoglobin; all animal hemoglobins were utilized equally well (2). *N. gonorrhoeae* is efficient in hemoglobin and heme utilization and all tested strains possess *hmbR*-related sequences (5, 2). However, the nucleotide sequence of MS11 *hmbR* gene contains a stop codon suggesting that another receptor, most probably Hpu, is responsible for Hb utilization in some *N. gonorrhoeae* isolates (2).

Peptide scanning approach was utilized in order to identify domains of the HmbR receptor and the hemoglobin molecule that interact with each other. A set of peptides derived from amino acid sequences of hemoglobin a and b chains and the HmbR protein were synthesized and their ability to inhibit hemoglobin utilization was determined. Two out of twenty two HmbR-derived peptides and two out of seventeen peptides originating from similar regions of Hb a and b chains, significantly inhibited hemoglobin utilization in the growth promotion plate assay. This results are another proof that the HmbR receptor recognizes globin part of hemoglobin molecule. Deletion analysis of the HmbR-surface exposed loops is currently carried out in order to corroborate the results of peptide scanning.

E. coli cells expressing the HmbR protein were not fully capable to use hemoglobin as an iron source indicating that additional *N. meningitidis* proteins are involved in hemoglobin utilization (1). In order to identify additional proteins involved in Hb utilization we have reconstituted the hemoglobin utilization system in *E. coli.* A *N. meningitidis* cosmid library was introduced into a heme-requiring mutant of *E. coli* expressing the HmbR protein. Transformants were plated onto Hb-supplemented media. Cosmids from five colonies which able to use hemoglobin as both porphyrin and iron source were further studied. When retransformed into *E. coli hemA,* cosmids allowed growth on Hb only in the presence of a *hmbR*-expressing plasmid. Nucleotide sequence analysis of DNA fragments subcloned from one of the Hb-positive cosmids identified three ORFs homologous to *Ps. putida, E. coli* and *H. influenzae exbB, exbD* and *tonB* genes. In order to understand the role these genes play in hemoglobin utilization, a *tonB* mutant of *N. meningitidis* was constructed. Preliminary data indicate that utilization of transferrin, lactoferrin, hemoglobin and haptoglobin-hemoglobin complexes as sole sources of iron is a TonB-dependent in *N. meningitidis.* Conversely, the *N. meningitidis tonB* mutant was still able to use heme indicating the existence of TonB-independent heme-uptake system in *Neisseriae*.

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Cloning, sequencing and genetic characterization of *tonB-exbB-exbD* genes of *Neisseria gonorrhoeae*

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N. gonorrhoeae is able to utilize efficiently iron (Fe) bound to transferrin (TF) and lactoferrin (LF). To understand the mechanisms used by gonococci to scavenge Fe from TF and LF, we isolated chemical mutants of strain FA19 that were unable to grow with Fe bound to either TF(TF^-) or LF(LF^-) or to both TF and LF($[TFLF]^-$)(3). The TF^--specific or LF^--specific mutants lacked receptor activity for TF or LF respectively(3), and they have been used successfully in cloning receptors for TF and LF(1,2). The $(TFLF)^-$ mutant designated *tlu* bound both TF and LF well, but the biochemical defect in the mutation was not known(3). In this report, we characterized the *tlu* loci through cloning, sequencing, transposon mutagenesis, and phenotypic studies.

An approach described previously was used to clone a wild type gene that is able to repair the defect in *tlu* mutations(1,2). A $(TFLF)^-$ mutant strain was transformed with pools of wild type FA19 DNA cloned in pBluescript, selecting for TF^+ transformants. A transforming clone was identified containing 404 bp gonococcal insert. By chromosome walking, 3488 bp of contiguous FA19 DNA were cloned. Nucleotide sequence identified three contiguous open reading frames (ORF) designated as ORF1, ORF2 and ORF3 that are arranged in tandem. The first two ORFs are separated by 66 bp, and the third ORF is situated 18 bp downstream of the second ORF. The deduced amino acid sequence of the 852 bp long ORF1 encoded a protein that exhibited 26% identity with the TonB protein of *E .coli* (5), whereas the 648 bp long ORF2 and 435 bp long ORF3 was 27% and 36% identical to that of of the ExbB and ExbD protein respectively of the same organism (4). The three ORFs are situated on a 4.5 kb gonococcal *Hinc* II fragment, and were able to repair six independently derived *tlu* mutations by DNA contained within these ORFs.

Each of the three ORFs in Bluescript vector were mutagenized with the interposon omega, and were then transformed into FA19. Each of the resulting mutants lost the ability to utilize either TF, LF, or human hemoglobin (Hgb), but could utilize hemin or Fe-citrate for growth. ^{55}Fe uptake assays with ^{55}FeTF, ^{55}FeLF, ^{55}Fe-hemin or ^{55}Fe-citrate showed that the same strains were unable to take up ^{55}Fe from TF or LF but internalized ^{55}Fe from hemin or citrate. However, the mutants bound TF, LF and Hgb normally as determined by dot blot assay.

Thus, the similarity in amino acid sequence homology and the pleiotropic nature of Fe related phenotype between the gonococcal ORF1-ORF2-ORF3 complex and *E. coli* TonB-ExbB-ExbD proteins suggest that ORF1, ORF2, and ORF3 represent *ton*B, *exbB*, and *exbD* genes in gonococcus. Moreover, the results show unambiguously that the TF, LF and Hgb receptors, but not the heme and citrate-iron uptake pathways, are dependent on the TonB system.

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Genetic and serological analysis of lactoferrin receptors in the Neisseriaceae: evidence for the antigenically conserved nature of LbpB

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The utilization of iron from transferrin (Tf) and lactoferrin (Lf) by pathogens in the *Neisseriaceae* is believed to occur via a multicomponent pathway which includes host specific Tf (TbpA+B) and Lf (LbpA+B) receptors on the organism's cell surface, a periplasmic iron binding protein (FbpA) which shuttles the ferric iron to the cytoplasmic membrane, and cytoplasmic membrane components which provide energy for the active transport of the Fe from the extracellular milieu to the periplasm (TonB with associated proteins Exb and ExbD), and across the cytoplasmic membrane (FbpB and FbpC) into the cytoplasm. The outer membrane receptor complex is believed to consist of a lipidated-hydrophilic component (TbpB and presumably LbpB), and a membrane spanning component (TbpA and LbpA) which may act like a gated pore, to allow entry of the Fe into the periplasm. The TbpA and LbpA proteins of a number of different organisms are highly homologous, and are 43% identical for *Neisseria meningitidis*.

The recently described LbpB molecule has been shown to reside in the human pathogens *N. meningitidis* (strains M982 and P3006), *Moraxella catarrhalis* (strain 4223) and the bovine pathogen *Moraxella bovis* (strain n112). In each case, the growth and binding characteristics of these organisms Lf receptors were specific for the host Lf [1]. Previously, we have demonstrated that the *lbpA* gene was the present in all clinical isolates of *N. meningitidis* and *N. gonorrhoeae*, as well as being present in virtually all commensal *Neisseria spp.* [2]. However, there is neither genetic or biochemical evidence which examines the ubiquity of the *lbpB* gene in pathogenic *Neisseria spp*. Therefore, we used a specific *lbpB* gene probe to examine, by Southern blot analysis, several pathogenic *N. meningitidis* and *N. gonorrhoeae* strains for the presence of this gene. The presence of the *lbpB* gene product was confirmed in these strains using polyclonal antisera against the *N. meningitidis* P3006 LbpB. We were also able to include a limited number of clinical gonococcal isolates in this analysis that were unable to utilize Lf as a sole iron source.

Several reports, to date, have shown that TbpB proteins (formerly designated Tbp2) from different meningococcal strains are quite heterogeneous in both molecular weight and serological cross-reactivity. However, there is currently no information about LbpB antigenic heterogeneity (or lack thereof). Our analysis consisted of using the western blot technique to test the ability of rabbit polyclonal antisera derived against either the *N. meningitidis* (strain P3006) LbpB, the *M. catarrhalis* (strain 4223) LbpB, or the *M. bovis* (strain n112) LbpB protein to react with the LbpB molecules from different *N. meningitidis, M. catarrhalis* or *M. bovis* isolates. Our results have indicated that, unlike the TbpB molecule, the molecular weight of the LbpB is approximately identical in all strains tested. In addition, the sera derived against the *N. meningitidis* (strain P3006) LbpB molecule reacted with all meningococcal strains tested, and also reacted weakly with the *M. catarrhalis* (strain 4223) and *M. bovis* (strain n112) LbpB molecules. Sera against the either the *M. catarrhalis* (strain 4223) or *M. bovis* (strain n112) LbpB molecules demonstrated varying degrees of reactivity with a number of meningococcal

isolates. In addition, we demonstrate the ability of antisera against the LbpB molecule to interact strongly with whole cells grown in iron restricted conditions, and only weakly with cells grown in an iron sufficient environment. These observations suggest that LbpB is surface exposed, is expressed when free iron is limiting, and is a potentially useful vaccine target due to its conserved antigenic nature and ubiquity among pathogenic strains.

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Characterization of the interaction between *Neisseria meningitidis* transferrin binding proteins and transferrin by gel filtration and surface plasmon resonance

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Neisseria meningitidis produces two iron repressible transferrin binding proteins; Tbp1 (100kDa) and Tbp2 (65-85kDa depending on strain) (1, 2). Both proteins are surface-exposed and are associated with the sequestration of iron from human serum transferrin (HST 80kDa) (3, 4). Iron uptake from this source requires the expression of Tbp1 and Tbp2 suggesting that these proteins interact and together form the functional HST receptor (5). In this study gel filtration and surface plasmon resonance (SPR) (6) have been used to characterise the association between Tbp1 and Tbp2, and the binding of HST by meningococcal Tbps.

Tbp1+2 was isolated from iron limited cultures of *N. meningitidis* using HST-Sepharose affinity chromatography, as described previously (7). Separate Tbp1 and Tbp2 were obtained by ion exchange chromatography using a Mono S column (Pharmacia) on FPLC. Analysis of affinity purified Tbp 1+2 and recombined separate Tbp1 and Tbp2 by gel filtration on a Superose 12 column (Pharmacia) indicated the formation of a ~300kDa complex. This complex bound HST resulting in a mass increase estimated at ~100kDa.

Separate Tbp1 had an apparent molecular weight of ~200kDa on gel filtration, suggesting the formation of a dimer. This complex bound HST and the resultant species had a molecular weight of ~300kDa. Indicating that Tbp1 bound a single molecule of HST. Thus it appears that the intact Tbp1+2 receptor and putative Tbp1 dimer each bind HST in a 1:1 ratio.

Similar analysis of purified Tbp2 by gel filtration indicated the formation of~400-600kDa multimers. These structures were absent from native mixtures inferring that the interaction between Tbp1 and Tbp2 may prevent their formation. The high and variable molecular weight of such aggregations indicates that they may be of non specific-structure, possibly resulting from the inherent adhesive nature of lipidated Tbp2.

The interaction between Tbps and HST has also been analysed by SPR using the Biacore X biosensor (Pharmacia). HST immobilised on the dextran matrix by amine coupling bound Tbp1 forming a highly stable complex. Tbp2 also bound to HST although the resultant complex underwent rapid dissociation. Immobilised HST was treated with Tbp1 until no further binding was observed. At this point resonance data indicated that each molecule of HST bound more than 1 molecule of Tbp1. The HST-Tbp2 interaction was examined in the same way and results suggested a 1:1 ratio of binding. Prior saturation with Tbp1 did not inhibit subsequent Tbp2 binding and appeared to stabilise the resultant complex. These results indicate that Tbp1 and Tbp2 bind separate distinct regions of HST and may interact following this association. This is

consistent with gel filtration results indicating that the functional HST receptor is formed by two molecules of Tbp1 and one molecule of Tbp2.

It has been shown that gonococci expressing only Tbp2 preferentially bind iron loaded (HOLO) HST over iron free (APO) HST (8). This has been demonstrated using the isolated high molecular weight form of meningococcal Tbp2 in an ELISA based study. Further gel filtration and SPR studies are in progress to investigate the kinetics and stoichiometry of APO/HOLO HST binding by meningococcal Tbps.

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Analysis of respiration linked nitrite reduction in *Neisseria gonorrhoeae*: AniA, the major anaerobically induced outer membrane protein, is probably not the terminal nitrite reductase.

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Neisseria gonorrhoeae (GC) is one of the primary causes of pelvic inflammatory disease (PID). Gonococcal PID is most likely an anaerobic infection as half of these cases are mixed infections with GC isolated along with obligate anaerobes (1). In order to grow under anaerobic conditions, GC uses nitrite as a terminal electron acceptor for respiration (2).

Previously, the outer membrane lipoprotein AniA (Pan1) was identified (3,4) and the *aniA* gene cloned and sequenced (5). Recent database searches indicate that AniA has 30-35% homology with copper-containing nitrite reductases (NiR) from other species. Outer membranes containing AniA reduced nitrite when methyl viologen was added as a gratuitous electron donor. In contrast, NiR from other species are typically periplasmic or bound to the cellular membrane (6), nor have any terminal reductases been found in the outer membrane in other bacterial species. Even though AniA appears to be capable of reducing nitrite, it is hard to envision that AniA could function as a respiration linked NiR with an outer membrane location.

In order to differentiate respiration linked NiR activity from total NiR activity, a physiological electron donor system was contrasted with an artifical gratuitous system. When glucose was added to measure respiration linked to the oxidation of NADH, there was a ten-fold increase in NiR activity in anaerobically vs. aerobically grown GC. If methyl viologen was used, there was no difference in activity of aerobically grown GC compared to the respiratory linked NiR activity, while there was a 300-fold increase in total NiR activity in anaerobically grown cells. This strongly suggested two pools of NiR activity, one with access to an internal supply of reducing equivalents, the other without access. The presence of NiR activity in aerobically grown GC indicates that there is an enzyme other than AniA that is responsible for this reductase activity, as there is no detectable AniA protein or *aniA* mRNA transcript in aerobically grown cells (5).

Osmotic shock was used to separate periplasmic proteins from the rest of the cell. The periplasmic fractions from aerobic and anaerobically grown GC showed similar NiR specific activity. Again, as aerobically grown GC do not contain detectable levels of AniA or *aniA* mRNA transcript (5), this similar activity in the two periplasmic fractions is most likely due to a protein separate from AniA.

In order to study the regulation of the *aniA* gene, the *aniA* promoter was cloned into the pLES94 construct and transformed into GC (7). This resulted in an *aniA*::*lacZ* fusion in single copy in the GC chromosome at a site other than the *aniA* site. RNase protection studies confirmed the pLES940 construct accurately reflects *aniA* promoter activity from both the gearbox and sigma^{70} promoters, as well as accurately reflecting the level of *aniA* expression from each individual

promoter. This construct was then used to show that under aerobic conditions, GC NiR activity increased 4-fold at the onset of stationary phase while the β-galactosidase reporter under control of the *aniA* promoter did not show any change in level of activity. This differential regulation between NiR activity and β-galactosidase activity underscored the likelihood that a second enzyme was responsible for the increase in NiR activity.

In order to track NiR activity in GC separate from AniA expression, a fusion protein with the FLAG™ peptide covalently bound to the to the C-terminus of AniA was constructed and transformed into GC. Aerobic and anaerobically grown GC containing the fusion were tested for NiR activity and equivalent cell amounts were analyzed by SDS-PAGE and a Western blot probed with anti-FLAG™ monoclonal antibodies. While both groups of cells had NiR activity, there was no detectable FLAG™ peptide in the aerobically grown cells, indicating that a protein other than the AniA::FLAG™ fusion was responsible for the nitrite reduction in these cells.

While at first glance AniA's function appears to be nitrite reduction, further studies into nitrite reduction in GC have led to the conclusion that there is a second enzyme that is responsible for respiration linked nitrite reduction. This is based on the presence of NiR activity in aerobically grown cells while there is no AniA or *aniA* mRNA transcript in these cells, as well as the differential regulation between NiR activity and β-galactosidase activity.

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Gonococcal FrpB: a possible role in siderophore uptake

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FrpB is an iron-regulated outer-membrane protein common to both *Neisseria gonorrhoeae* and *Neisseria meningitidis*. This protein shares homology with the TonB-dependent class of outer-membrane proteins common among Gram negative species (1), the most closely related proteins being CopB of *Moraxella catarrhalis* (71% similarity) (2) and the hemin receptor, HemR of *Yersinia enterocolitica* (48% similarity) (3). Much labor has gone into trying to determine the function of FrpB, but to date, it has remained elusive. Initial experiments indicated that a FrpB-deficient strain of *Neisseria gonorrhoeae* is somewhat impaired in its ability to utilize iron from heme and possibly lactoferrin, but further studies revealed that this decreased ability may actually be due to non-specific membrane perturbations in the mutant strain (1; B. Stone, unpublished data).

During the initial sequencing of *frpB*, the start of an open reading frame (about 100 base pairs) was identified directly downstream. A GCG database search revealed this sequence to share homology with various periplasmic siderophore transporters (Beucher, unpublished data). This encouraged us to sequence further downstream of *frpB* in the hope that some clues about FrpB function could be gained.

Sequencing downstream of *frpB* revealed at least three open reading frames (orfs). Each has sequence similarity to components of siderophore uptake systems, especially the phenolate-siderophore uptake systems. The *orf1* deduced amino acid sequence shares extremely high degrees of sequence similarities with periplasmic phenolate-siderophore transporters, especially CeuE, the *Campylobacter coli* enterobactin periplasmic transporter (4), and FatB, the *Vibrio anguillarum* anguibactin periplasmic transporter (5). Additionally, the *orf1* deduced amino acid sequence contains the "signature sequence" present in all known periplasmic siderophore transporters (6). The *orf2* and *orf3* deduced amino acid sequences share strong degrees of sequence similarities with members of the cytoplasmic siderophore transporter family, including CeuB and C (4), and FatC and D (5). Because of the homologies between the three open reading frames and components of siderophore transport systems, we hypothesize that these open reading frames may play a role in the uptake of iron from siderophores in *Neisseria gonorrhoeae*. The presence of these open reading frames located directly downstream of *frpB* implicates siderophore uptake as a possible role of FrpB.

Although it has long been believed that the *Neisseriaceae* produce no siderophore of their own (7), the gonococcus is able to scavenge iron from the siderophore aerobactin from co-existing bacteria (1), as well as from various human carrier proteins (8-10). Mutation of *frpB* did not affect aerobactin utilization (1), but other siderophores were not tested carefully. Studies are presently underway in the laboratory to determine whether *frpB* is transcriptionally linked to the downstream orfs and whether FrpB and Orf1, Orf2, and Orf3 play a role in siderophore uptake.

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A phase varying, hemoglobin-binding outer membrane protein from *Neisseria gonorrhoeae*

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The majority of *in vitro* variants of *Neisseria gonorrhoeae* were unable to use human hemoglobin as the sole source of iron (Hgb-), but a minor population were able to do so (Hgb+). This minor population grew luxuriously on hemoglobin, bound biotinylated hemoglobin and expressed a novel outer membrane protein of 89 kDa. The ability to use hemoglobin for growth apparently was a phase varying phenomenon. Among all strains tested, the Hgb- to Hgb+ variation rate was 0.08 to 2.15 variants per 1000 CFU. The 89 kDa protein was regulated by levels of iron present in the medium and was purified on immobilized hemoglobin.

The N-terminal amino acid sequence of the 89 kDa protein revealed identical amino acids, from position 2 to 16, to HpuB, an 85 kDa iron-regulated hemoglobin-haptoglobin utilization outer membrane protein of *Neisseria meningitidis*. Isogenic mutants of FA19 and FA1090 constructed by allelic replacement using a meningococcal *hpu*::mini-Tn3erm construct (1) no longer expressed the 89 kDa protein. Mutants could not utilize hemoglobin to support growth but still grew on heme. Thus, the gonococcal HpuB homologue is a functional hemoglobin receptor. Using chromosomal DNA from isogenic mutant of FA19, additional insertional mutants were obtained from hemoglobin utilizing variants of several clinical gonococcal strains. These mutants also lost their ability to utilize hemoglobin. The gene for another hemoglobin binding protein, HmbR, has been found in *N. meningitidis* and *N. gonorrhea* MS11 (2, 3). Southern blot hybridization of hemoglobin utilizing FA19 and FA1090 DNA with MS11 *hmbR* demonstrated that these two strains also have the gene encoding HmbR. However, insertional inactivation of the *hmbR* gene did not affect the hemoglobin utilization of either FA19 or FA1090. Thus, while gonococci may have genes for two different hemoglobin binding proteins, the 89 kDa HpuB homologue is essential for the utilization of hemoglobin.

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Diversity, topology, and functional domain mapping of gonococcal transferrin-binding proteins

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The gonococcal transferrin receptor consists of two iron-regulated proteins, Tbp1 and Tbp2. Tbp1 shares homology with a family of outer membrane receptors required for the energy-dependent uptake of ferric siderophores and vitamin B_{12} in *E. coli* (1). Models of membrane topology for these receptors have been generated using a variety of computer algorithms and domain mapping techniques (2, 3). We have generated a hypothetical model of Tbp1 using both computer predictions and sequence heterogeneity observed among three gonococcal sequences and two meningococcal sequences. Conserved stretches of amino acids are expected to be buried in the membrane while variable stretches are localized in exposed loops. We raised polyclonal antibodies against peptides in predicted surface-exposed domains and tested these antibodies for their ability to bind to the gonococcal cell surface, to block transferrin access to the receptor, and to initiate complement-mediated killing of wild-type gonococci. While several antibodies raised against hypothetically exposed loops of Tbp1 reacted against denatured Tbp1 in western blots, only one bound to whole gonococci. The domain to which this antibody was raised is located in the amino-terminal third of the proposed Tbp1 model. In preliminary experiments, this antibody also inhibited access of ferrated transferrin to the receptor and initiated complement-mediated killing of wild-type gonococci. We conclude from these experiments that a domain in the amino-terminal third of Tbp1 is surface-exposed, is important for interaction with ferrated transferrin, and potentially a site for binding of bactericidal antibody.

Gonococcal Tbp2s are more diverse at the predicted protein level than are gonococcal Tbp1s. We have compared five gonococcal Tbp2 sequences (4) with the published meningococcal Tbp2 sequences (5, 6). Several conserved stretches can be identified in an alignment of all 10 Tbp2 sequences; these domains may be important for some aspect of Tbp2 function, localization or stability. By expressing truncated versions of Tbp2, we have delimited a minimal transferrin-binding domain of gonococcal Tbp2, which extends through the amino-terminal half of the protein. Two stretches of conserved residues are contained within the transferrin-binding domain, thus they may be important for optimum ligand binding. Conserved and variable domains of gonococcal Tbp2s will be presented in the context of observations of functional constraints made for Tbp2s from other pathogenic bacteria (7, 8).

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Evidence for a bi-lobed structure for meningococcal transferrin binding protein B

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Members of the Neisseriaceae utilize a cell-surface receptor that binds transferrin (Tf) as an initial step in the iron acquisition process. This transferrin receptor is comprised of two outer membrane proteins, transferrin binding protein A (TbpA) and transferrin binding protein B (TbpB). Comparative analysis of the TbpA sequence predicts an integral transmembrane protein which may act as a gated pore for iron (1). TbpB is predicted to be a peripheral outer membrane lipoprotein anchored to the membrane via a N-terminal-linked fatty acid (1). It has been proposed that there is a conserved mechanism of iron acquisition amongst species containing transferrin receptors which predicts a conserved receptor-ligand interaction. Previous studies have demonstrated that regions of TbpB from *N. meningitidis* and *A. pleuropneumoniae* involved in binding to Tf can be localized to the N-terminal portion of TbpB (2,3). As well, TbpB from human pathogens have been shown to bind to the C-terminal lobe of human transferrin (hTf) (4). However, the concept of a conserved receptor-ligand interaction is difficult to resolve with the demonstration that TbpB from several bovine pathogens binds to the N-terminal lobe of bovine Tf (5).

As part of further elucidation of the mechanism of Tbp-mediated iron procurement from Tf, the specific interactions between the TbpB receptor component of meningococcus and human transferrin (hTf) were investigated. Amino acid sequence alignment of the N-terminal and C-terminal halves of strain M982 TbpB revealed regions of identity, implying that a duplication event gave rise to sequences present in both the N-terminal and C-terminal halves. To further investigate the character of these two regions of TbpB, a series of M982 N- and C-terminal TbpB truncations and N- and C-terminal fusions were utilized to identify regions of TbpB required for interaction with hTf (3).

Recombinant TbpBs expressed in *Escherichia coli* consisted of the N-terminal half of TbpB; the C-terminal half of TbpB; incremental C-terminal deletions of the N-terminal half of the molecule; and maltose binding protein fusions of the N-terminal and C-terminal TbpB halves. After confirmation of expression of the TbpB truncations and fusions by western blot analysis of whole cell lysates, the ability to bind hTf was investigated. Using whole cell lysates as a source of the TbpB truncations, solid phase and affinity isolation assays were performed. These two methods of analysis provided corroborating results where both the N-terminal and C-terminal halves of M982 TbpB were shown to bind hTf. Deletion constructs containing smaller portions of the N-terminal region were unable to bind hTf in either assay. Chimeric hTf/bTf-N/C lobe transferrins were also utilized to localized the binding determinants for these halves of TbpB to the individual lobes of hTf.

The conservation of sequence between the two halves of TbpB, combined with the ability of both halves to bind hTf, provide evidence that TbpB arose from a gene duplication event. This also has implications regarding TbpB structure. As each half of TbpB can independently bind hTf, TbpB may consist of two lobes, each with a distinct binding region for Tf. This proposed bi-

lobed structure of TbpB may be a conserved feature of TbpB from various species, and the ability to demonstrate this may be dependent on the nature of the assay conditions utilized.

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Conserved interactions between heterologous TbpB-TbpA pairs and Tf.

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In response to iron limitation, bacteria in the families *Pasteurellaceae* and *Nesseriaceae* synthesize outer membrane receptor complexes which bind host transferrin (Tf) as the first step in an iron acquisition pathway. The receptor complex consists of Tf binding proteins B (TbpB) and A (TbpA) (1). While both TbpB and TbpA of the human pathogens *Neisseria meningitidis* and *Haemophilus influenzae* bind human Tf (hTf) independently in solid phase binding assays, affinity isolation of TbpB is dependent on the presence of TbpA in an affinity isolation assay using hTf-Sepharose. In addition, both TbpB and TbpA are required for optimal acquisition of hTf-bound iron supplied as a sole iron source in an *in vitro* growth assay (2,3). Therefore, we hypothesized that a specific interaction was occurring between TbpB and TbpA or that a conformational change in hTf resulting from TbpA binding increases TbpB binding to Tf to detectable levels. Furthermore, we hypothesized that this interaction(s) is conserved among the human pathogens *H. influenzae* and *N. meningitidis*.

In an attempt to further investigate this putative TbpB-TbpA interaction, we decided to examine whether it was present when heterologous TbpAs and TbpBs were studied. Thus we affinity isolated TbpA from *N. meningitidis* and *H. influenzae* isogenic mutant strains (2,3) and TbpA from *M. catarrhalis* using selective binding and/or elution conditions (4) and subsequently assessed their ability to facilitate isolation of TbpB from *N. meningitidis* or *H. influenzae*. These studies demonstrated that heterologous TbpAs from human pathogens could facilitate isolation of both TbpBs indicating that this is a conserved interaction.

To further investigate whether this phenomenon was due to TbpA-TbpB interaction or modification of Tf binding to TbpB, we examined the ability of TbpA from *N. meningitidis* to affinity isolate TbpBs from the porcine pathogen *A. pleuropneumoniae* and the bovine pathogen *H. somnus* using an hTf-Sepharose affinity matrix. The *H. somnus* and *A. pleuropneumoniae* TbpBs were isolated by the hTf-Sepharose matrix in the presence of the *N. meningitidis* TbpA, but not in its absence. Identical results were obtained using apo-Tf, indicating that the interaction is not dependent on the iron-loaded state, and the corresponding conformation, of Tf. Collectively, these results suggest a TbpA-TbpB interaction is responsible for this phenomenon, rather than modulation of Tf structure by TbpA.

In an attempt to localize regions of TbpBs involved in interactions with TbpA or hTf, the ability of a series of chimeric *A. pleuropneumoniae-N. meningitidis* TbpBs to affinity isolate on hTf in the presence and absence of the *N. meningitidis* TbpA or on pTf in the presence or absence of the *A. pleuropneumoniae* TbpA was examined. Several chimeric TbpBs were identified that were able to affinity isolate in the presence of the heterologous TbpA. A detailed analysis of these results will be presented and discussed.

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Specificity of the gonococcal heme transport system

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Neisseria gonorrhoeae can utilize free heme as a source of Fe for growth; however, little is known concerning the mechanisms involved in heme transport. We have characterized the binding and accumulation of heme by *N. gonorrhoeae* and defined the specificity of the gonococcal heme receptor. We have also initiated studies to determine if *N. gonorrhoeae* produces a hemolytic-like-activity. In many pathogenic organisms a hemolysin or cytotoxin appears to function in the lysis of erythrocytes in vivo, resulting in the liberation of heme which may then be utilized as an iron source. Our results indicate that whole cell extracts obtained from *N. gonorrhoeae* F62, FA19, and MS11 are capable of lysing sheep red blood cells. Thus, the gonococcus may possess the ability to lyse erythrocytes in vivo resulting in the liberation of heme; the iron from heme could then be transported via the gonococcal heme transport system. Our results indicate that a common receptor which recognizes heme (through the PPIX ring) is involved in binding both heme and hemoglobin. Binding of radiolabeled heme was shown to be inhibited by the addition of heme, hematoporphyrin, or hemoglobin, but not by ferric citrate. Thus *N. gonorrhoeae* may utilize at least 2 receptors for the binding of heme-containing compounds; the putative hemoglobin receptor which binds both heme and hemoglobin, and a second receptor specific only for heme. Following the interaction of heme with the gonococcal hemin receptor(s), ^{59}[Fe] from radiolabeled heme is taken up into the cell at a constant rate by an energy dependent mechanism. We also found that the majority of ^{59}[Fe] from heme was associated with the gonococcal periplasmic ferric binding protein, FbpA. We did not detect the uptake of ^{14}[C] from radiolabeled heme indicating that the PPIX ring is not transported into the cell. Taken together, our results indicate that heme binds to gonococcal outer membrane receptors through the PPIX ring, and following binding, iron is removed and transported into the cell where it is associated with the periplasmic ferric binding protein, FbpA.

Molecular analysis of *lbpAB* encoding the two component meningococcal lactoferrin receptor.

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Pathogenic Neisseria are able to acquire Fe from lactoferrin (LF), transferrin (TF), hemoglobin (Hb) and Hb complexed to haptoglobin (Hb-Hp) (1-6). Acquisition of Fe from TF, Hb and Hb-Hp inv
olves the production of a bipartite receptor composed of a specific TonB dependent transport protein and an accessory lipoprotein (7, 8) . Acquisition of Fe from LF is associated with the production of the TonB dependent outer membrane protein, LbpA (4).

We cloned and analyzed *lbpA* from *N. meningitidis* DNM2. The predicted amino acid sequence of LbpA suggests that LbpA is an outer membrane protein with a 24 amino acid leader peptide and a signal peptidase I cleavage site. At the amino acid level LbpA shares a high degree of similarity with TonB dependent outer membrane receptors, suggesting that LbpA is a member of this family of high affinity transporters. The LbpA protein of DNM2 appears to be highly conserved sharing 99% identity with IroA (3) and 95% identity with the LbpA identified by Pettersson *et al* (9) .

5' to *lbpA* we discovered an open reading frame (ORF) which we designated *lbpB*. The predicted amino acid sequence of the putative LbpB is highly homologous to Tbp2, the lipoprotein component of the TF receptor, and may encode the lipoprotein component of the LF receptor.

Fe-availability controls the expression of proteins required for acquisition of Fe. Classically Fe repression occurs at the level of transcription and is mediated by the transcriptional repressor, Fur. A Fur homologue has been identified in the Neisseria (10) and RNA dot blot analysis indicates that expression of *lbpA* is regulated by Fe at the level of transcription. RT-PCR analysis confirms that regulation of *lbpA* occurs at the level of transcription and suggest that *lbpA* and *lbpB* are co-transcribed on a polycistronic mRNA.

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Transcriptional regulation of pilC2 of *Neisseria gonorrhoea*e: Response to oxygen availability and evidence for growth phase regulation in *E. coli*

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The type 4 pilus of *Neisseria gonorrhoeae* is a predominant surface antigen which facilitates adhesion to host target cells (1), an essential event in gonococcal infection. *pil*C2 encodes a 110 kDa pilus tip-located adhesin (2) that is involved in pilus-mediated adherence to human epithelial cells in culture, pilus assembly and natural competence for DNA transformation (3). Luciferase activity directed from a chromosomal *pil*C2::*lux*AB transcriptional fusion was reduced approximately 3-fold when cells were grown anaerobically versus aerobically. We observed a concomitant reduction in gonococcal piliation, by electron microscopy, and reduction in their ability to adhere to ME-180 human epithelial cells when bacteria were grown in the absence of oxygen. Additionally, we present evidence for growth phase regulation of the gonococcal *pil*C2 gene in *E. coli*, determining that all sequences necessary for growth phase regulation are contained on a 118 bp *pil*C2 fragment. Expression from the minimal *pil*C2 fragment fused to *lac*Z in single-copy in *E. coli* was induced 2-fold as cells exited exponential growth. Surprisingly, induction was independent of an intact *rpo*S gene which encodes the starvation induced sigma factor RpoS. In conclusion, we have demonstrated that *pil*C2 is both positively and negatively regulated at the level of transcription, with an overall change in expression of approximately 6-fold. This regulation is most likely relevant to physiological conditions within the human host which influence gonococcal infections.

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Sequence analysis of the structural *tbp*A gene: protein topology and variable regions within neisserial receptors for transferrin iron acquisition

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The meningococcal transferrin receptor is comprised of two transferrin-binding proteins, Tbp1 and Tbp2. Tbp1 is a 98 kDa transmembrane protein with only small variations in its molecular weight among strains, and was originally thought not to be exposed (1). Bishop et al. (2) raised murine monoclonal anti-Tbpl antibodies, one of which showed bactericidal activity and recognized a conformational epitope. This data suggests that Tbp1 is surface-exposed and immunogenic in humans and animals, and antibodies to their native structure could be bactericidal to homologous and heterologous strains. In order to assess at the molecular level the conservation of the Tbp1 protein, we obtained the complete sequence of the *tbp*A gene of the strain B385. The predicted B385 protein sequence was aligned with sequences with identities ranging from 43 to 93 percent including the closely related neisserial Lbps.

Variable Regions. The analysis of the multiple sequence alignment revealed large highly conserved areas and several well defined, smaller regions of sequence variability. Most differences among the meningococcal Tbp1s were localized to five regions of the mature protein, called VR1 (199-287), VR2(306-381), VR3(480-546), VR4(618-651) and VR5(681-708). The VRs may be associated with receptor specificity, since they show the highest sequence divergence between Tbps and Lbps, contain variable (and thereby thought to be exposed) regions, and also contain well conserved segments. By correlative analysis we were able to detect an overall 0.5 frequency of specific residues against a 0.3 frequency present in less variable segments which points out the contribution of such regions to the receptor specificity. The highest variability between Tbps and Lbps was found at VR1. We were able to detect a pair of well conserved cysteines (241 and 249) within this region (VR1), separated by a short and highly variable heptapeptide that is present in the Tbps but not in the Lbps.

VR3 resulted the most variable among Tbps. This region was also noticeable from the correlative analysis since we found that the Lbps have a deletion of 8-10 amino acids in this region compared to Tbps.

Topology model: We constructed a topology model for Tbp1 by applying the same principles that have been recognized for the structure of *E. coli* outer membrane proteins (3,4). The protein is thought to span the membrane 28 times, thereby exposing 14 hydrophilic loops to the outer surface, with VRs in the fifth, sixth, eighth, tenth and eleventh loops respectively.

Like FepA, and perhaps the other Ton-B dependent receptors (3), we postulate a similar gated channel model for Tbp1. As in the proposed topology model for Lbp (5) we propose large exposed loops, but we locate an additional loop in the outside layer of the membrane by assuming two additional membrane spanning sequences: a new one (14-21) at the N-terminus of

the mature protein, and by placing two b-strands in the region 183-198. Another major discrepancy refers to the sequence 113-SGAINEIEYEN-124, which we do not believe to form a transmembrane section, but to be included in a highly important extracellular loop (between b5 and b6), well conserved by structural or functional constraints in Tbp as well as in Lbp. We strongly believe that our model fits the Lbp as well, regarding the showed differences in some of the exposed regions.

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Molecular characterization of FrpB, the 77 kDa iron-regulated OMP

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Under iron limitation *Neisseria meningitidis* produces several extra outer membrane proteins (OMPs). To gain insight in the mechanism of iron acquisition by *N. meningitidis*, we are studying these iron-limitation inducible OMPs. The proteins are also studied to assess their vaccine potential. This report deals with the 77 kDa FrpB protein, the major iron-regulated OMP.

Monoclonal antibodies (mAbs) were raised against the FrpB protein of two different strains. The antibodies showed only a very limited cross-reactivity with various meningococcal strains (1). Most antibodies were bactericidal. The genes encoding FrpB from three different strains were cloned and sequenced. Comparisons between these *frpB* genes and the published gonococcal one (2) revealed that especially the region from amino acid residue 350 to 390 displays pronounced sequence variability. A topology model was constructed for FrpB, based, amongst others, on sequence comparisons. In this model, the most variable part corresponds to loop 7, the longest of the 13 predicted surface-exposed loops. By using synthetic peptides we could demonstrate that the epitopes of bactericidal mAbs are located within this loop. From five additional meningococcal strains, the parts of the *frpB* genes corresponding to this region were cloned and sequenced. The sequences can be divided into three groups, with a much higher similarity within the groups than between them.

We are investigating the possibility to direct the immune response away from the variable, immunodominant loop 7, towards other, more conserved loops. Mouse antisera were raised against synthetic peptides corresponding to the 13 loops predicted in the model and tested for binding to whole cells expressing FrpB. The highest binding was observed for antibodies directed against loops 6, 7 and 12 showing that in addition to the variable loop 7, other surface-exposed epitopes exist in this protein. An *frpB* mutant with a deletion of 38 amino acid residues in loop 7 was constructed and expressed constitutively in *N. meningitidis* strain H44/76 by placing it behind the *porA* promoter. The mutant protein was present in the outer membrane in amounts comparable to those of wild-type FrpB expressed from the same promoter. The deletion led to a strong increase of the binding of the mouse anti-peptide sera directed against loops 5, 6, and 12. This demonstrates that the immunodominant loop 7 does indeed shield other, more conserved loops. The ability of the *frpB* deletion mutant to induce antibodies against these other loops is now being investigated.

The FrpB protein showed homology to CopB of *Moraxella catarrhalis* and to the hemin-binding protein of *Yersinia enterocolitica.* Studies were performed to determine the function of the FrpB protein with the help of a knock-out mutant. The mutant showed no significant difference from its parental strain in iron acquisition from transferrin, lactoferrin, aerobactin, hemin or citrate. A minor effect on serum resistance was observed. The function of the protein is thus still unknown.

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Co-localization of the meningococcal transferrin binding proteins (Tbp1 and Tbp2) and evaluation of their relative roles in binding human transferrin.

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Apo-transferrin and holo-transferrin were separately conjugated to 15nm colloidal gold. Iron-restricted *Neisseria meningitidis* strain SD (B:15:P1.16) bound up to three-fold more holo-hTf than apo-hTF ($P<0.001$). The ability of a meningococcal mutant lacking either Tbp1 or Tbp2 to discriminate between apo and holo-transferrin was also investigated. There was no significant difference between the amount of gold-labeled apo-transferrin bound by the isogenic Tbp1 mutant (expressing Tbp2) and the parent strain ($p = 0.18$), whereas an isogenic Tbp2 mutant (expressing Tbp1) was significantly less effective at binding gold-labeled apo-transferrin ($P<0.001$). The isogenic Tbp1 and Tbp2 mutants and the parent strain all bound significantly more holo-hTf than apo-hTf, whereas the double "knock-out" mutant, failed to bind hTf irrespective of the iron-loading. In the isogenic mutants, Tbp2 was more effective in binding either apo or holo-transferrin than Tbp1.

Monoclonal antibodies against Tbp1 and Tbp2 were used to co-localize the transferrin-binding proteins on strain SD (B:15:P1.16). The ratio of Tbp1:Tbp2 was approximately 1:1. Tbp1 was occasionally observed in close proximity to Tbp2, but the two proteins were generally quite separate indicating they do not usually form a single transferrin receptor.

Study of human transferrin binding sites within the transferrin binding protein Tbp2 from *N. meningitidis* M982 using the pMAL expression system

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N. meningitidis M982 strain (B:9,P1.9) expresses a transferrin binding protein 2 (Tbp2) of 88 kDa [1]. The minimal human transferrin (hTf) binding domain was demonstrated to be localized in the N-Terminal part of the molecule (aa 34-350) [2]. A comparison between the N-terminal (aa 1-351) and the C-terminal (aa 352-691) regions of the Tbp2 amino acid sequence from four M982-like strains indicated that some stretches were repeated in the molecule suggesting that there may exist a symmetry in Tbp2 [3]. This observation implied that an hTf binding site may be present in the Tbp2 C-Terminal half but this remained to be demonstrated. The pMAL expression system (Biolabs) was used to verify this hypothesis as it allowed the purification of truncated forms of rTbp2 on amylose via the maltose binding protein (MBP) followed by the hTf binding analysis in non denaturing conditions. To validate the system, the full length Tbp2 (aa 2-691) was expressed as a fusion with MBP into the pMAL-c2 (cytoplasmic targeting) and pMAL-p2 (periplasmic targeting) vectors; no difference was observed in terms of hTf binding capacity. The PCR products encoding the N- and C-Terminus of M982 Tbp2 were cloned into pMAL-c2 and the MBP-rTbp2 fusions were characterized relative to their antigenicity and hTf binding ability. By using a chemiluminescent kit (Amersham), the C-Terminus fusion was shown to bind human transferrin on Western Blot; using the purified fusion proteins and the same sensitive detection, we demonstrated by dot blot that the hTf binding capacity of the C-Terminus was much more reduced than that observed with the N-Terminus and that the hTf binding activity located in the N-terminal half was also less than that of the full length Tbp2. Based upon this observation and along with the M982 Tbp2 sequence symmetry, ten other constructs were produced and characterized to localize more precisely the binding site(s) within the C-Terminus. The presence of this other binding site was further localized to the central region of the molecule and no hTf binding was visualized within the distal region as defined by this kind of expression system. The involvement of the C-terminal peptide repeated in the N-Terminus of M982 Tbp2 and common to the same homologous peptide in Tbp2 from *A. pleuropneumoniae* [5] was examined. Furthermore, the role of the three major hypervariable stretches within the Tbp2 central region (as previously described [4]) on the hTf binding ability of the full length molecule was investigated using deletion variants (Δ aa 388-396, Δ aa 418-476, Δ aa 499-521). Preliminary data suggested that a particular fragment around the aa 388-396 region may be involved in positioning correctly the C-Terminus and the N-Terminus into the full length Tbp2 for ensuring an efficient human transferrin binding and therefore it may perhaps function as an hinge between the two domains.

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Characterization of the meningococcal lactoferrin receptor

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Efficient iron acquisition is an important virulence factor for pathogenic bacteria, since the concentration of free iron in the human host is low. To overcome this problem, many bacteria produce small, iron-chelating compounds, called siderophores, which are able to scavenge iron very efficiently from the environment. The pathogenic *Neisseriae,* however, do not produce siderophores, but are able to acquire iron directly from host iron-binding proteins, such as transferrin in serum and lactoferrin on mucosal surfaces (1). Under iron limitation , the synthesis of several outer membrane proteins (OMPs), including receptors for transferrin and lactoferrin, is induced. We are studying the molecular mechanism of iron-acquisition from lactoferrin by *N. meningitidis.*

By screening a λgt11 gene library with monoclonal antibodies, we have cloned the *lbpA* gene, encoding the 102 kDa iron limitation-inducible lactoferrin receptor. Expression of this gene in *E. coli* allowed these cells to bind lactoferrin, but not to use lactoferrin as an iron source (2). Sequencing of the gene revealed that this lactoferrin receptor is highly homologous to TbpA, one of the two transferrin-binding proteins in the neisserial outer membrane. Moreover, some homology to TonB-dependent siderophore receptors of *E. coli* was found, suggesting the involvement of a TonB homologue in iron-acquisition from lactoferrin (3). The presence of homologues of TonB and its accessory proteins ExcB and ExcD in *N. meningitidis* could be demonstrated in Southern blotting experiments, and the corresponding genes were cloned.

Upstream of *lbpA,* an open reading frame was identified, tentatively designated *lbpB.* A band, probably corresponding to LbpB, could be detected with peroxidase-conjugated lactoferrin after blotting of outer membrane proteins of *Neisseria meningitidis* from SDS-polyacrylamide gels to nitrocellulose paper. The putative LbpB protein displays homology to TbpB, the second transferrin-binding protein in the neisserial outer membrane (4). Therefore, we assume that iron-acquisition from lactoferrin requires two distinct lactoferrin-binding proteins. At the moment we are cloning and sequencing the part of *lbpB,* corresponding to the N terminal part of the protein.

Immunological cross-reactivity studies and sequencing of the *lbpA* gene of another strain revealed a high degree of conservation. The variation was mostly restricted to a few cell surface-exposed loops in a proposed topology model. The protein is supposed to traverse the outer membrane 26 times in a beta-sheet conformation, exposing the most variable and hydrophilic parts to the outer surface (4). The variability of the most immunogenic loop, containing the epitopes of the available monoclonal antibodies, was studied in further detail after PCR amplification of the corresponding DNA fragment of several strains. Presently, we are verifying the proposed topology model for LbpA experimentally.

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