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Lactoferrin receptors in Gram-negative bacteria: Insights into the iron acquisition process

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Abstract

One component of the anti-microbial function of lactoferrin (Lf) is its ability to sequester iron from potential pathogens. To overcome this iron limitation, a number of Gram-negative bacterial pathogens have developed a mechanism for acquiring iron directly from this host glycoprotein. This mechanism involves surface receptors capable of specifically binding Lf from the host, removing iron and transporting it across the outer membrane. The iron is then bound by a periplasmic iron-binding protein, FbpA, and transported into the cell via an inner membrane complex comprised of FbpB and FbpC. The receptor has been shown to consist of two proteins, LbpA and LbpB. LbpB is bilobed lipoprotein anchored to the outer membrane via fatty acyl groups attached to the N-terminal cysteine. LbpA is a homologue of siderophore receptors, which consist of an N-terminal plug and a C-terminal beta-barrel region. We propose that the receptor proteins, LbpA and LbpB, induce conformational changes in human Lf (hLf) that lower its affinity for iron that binding by FbpA can drive the transport across the outer membrane, a mechanism shared with transferrin (Tf) receptors. The interaction between the receptor proteins and Lf is quite extensive and has been previously studied by using chimeric proteins comprised of Lf & Tf. In an attempt to evaluate the role of FbpA in the transport process, a series of site-directed mutants of FbpA were prepared and used to replace the wild-type protein in the iron acquisition pathway. The mutations were made in the iron-binding and anion-binding ligands of FbpA and were designed to result in altered binding properties. Protein crystallography of the iron-bound form of the Q58L mutant protein revealed that it was in the open conformation with iron coordinated by Y195 and Y196 from the C-terminal domain but not by the other iron-liganding amino acids from the N-terminal domain, H9 and E57. Replacement of the native FbpA in *Neisseria meningitidis* with wild-type or mutant *Haemophilus influenzae* FbpAs resulted in a defect in growth on Tf or Lf, suggesting that there may be a barrier to functional expression of *H. influenzae* FbpAs in *Neisseria meningitidis*. Thus mutants of the *N. meningitidis* FbpA are being prepared to replace wild-type protein in order to test their ability to mediate transport from hLf.

Introduction

Iron serves as a prosthetic group for proteins such as cytochromes, oxidases, catalases, and ribonucleotide reductase that perform critical functions within almost all living cells (Bergeron 1986). It also is a key component in oxygen transport proteins such as hemoglobin that are essential for maintaining adequate oxygen levels in the tissues of multi-cellular organisms. A specialized iron-binding glycoprotein, transferrin, is responsible for transporting iron throughout the body. The iron-loaded form of transferrin is bound by a specific receptor on cells requiring iron, triggering an endocytic process in which the iron is removed from transferrin within the endocytic compartment (Richardson & Ponka 1997). The apo form of transferrin is subsequently released from the receptor complex at the cell surface after the endocytic vesicle fuses with the cell membrane.

Transferrin is present in serum at concentrations ranging from 20 to 30 μM with its iron binding capacity normally only 30% saturated. Due to the high affinity of transferrin for iron, levels of free iron are very low even with the release of additional iron into

the extracellular compartment. On mucosal surfaces and at sites of infection the predominant iron-binding glycoprotein is lactoferrin, which binds iron with a greater affinity than transferrin, particularly under the acidic conditions that would exist at sites of infection (Sánchez *et al.* 1992). There is a relatively high level of amino acid identity between transferrin and lactoferrin that reflects the nearly identical overall structure of these two glycoproteins (Baker *et al.* 1998). These bilobed, monomeric glycoproteins possess structurally similar N-terminal and C-terminal lobes that consist of two domains separated a deep interdomain cleft. The site for binding iron and its coordinating bicarbonate anion is the base of the cleft. In structures of the iron-loaded forms of these glycoproteins, the two domains are in close opposition (the closed conformation) whereas there is a variable degree of separation of the domains in the apo forms of the proteins (Baker *et al.* 2002). These results suggest that the iron-free lobe may fluctuate between the open and closed forms, although it likely would be predominantly in the open form.

In addition to its iron-withholding capabilities, lactoferrin possesses other potential antibacterial mechanisms (Chierici 2001) and is a source of a cationic bactericidal peptide that can be released from the N-terminus by proteolytic cleavage (Nibbering *et al.* 2001). There is an ever increasing range of functions attributed to lactoferrin that involve modulation of host cell activities in which the mechanisms are still being explored (Brock 2002), but are not primarily based on its iron-binding properties. Clearly this protein has evolved substantially from its primordial iron-withholding function.

The presence of transferrin or lactoferrin in substantial concentrations in the extracellular compartment within the body or on mucosal surfaces effectively maintains the level of free aqueous iron below that required to support microbial growth (Finkelstein *et al.* 1983). Many bacteria are capable of expressing high affinity iron uptake systems that are capable of acquiring iron from transferrin and lactoferrin, and thus overcome the iron-restricted environment of the host. The synthesis and secretion of small iron-chelating molecules (siderophores) that are capable of competing with the host iron-binding proteins for iron is the primary mechanism of iron acquisition in many species of bacteria (Braun & Braun 2002). In Gram-negative species, the resulting iron-siderophore complex is bound by cognate outer membrane receptor that mediates the transport of the complex into the periplasmic space. A specific periplasm to cytoplasm branch of the pathway subsequently transports the iron-siderophore complex into the cell.

An alternative mechanism that involves direct binding of the host glycoproteins by surface receptors is found in the highly host-adapted bacterial species in the families Neisseriaceae and Pasterurellaceae (Gray-Owen & Schryvers 1996). The surface receptor complex removes iron directly from the host glycoprotein and transports iron across the outer membrane to the periplasmic space where it is bound by a specific binding protein, FbpA. FbpA is structurally equivalent to the individual lobes of transferrin and lactoferrin and coordinates the ferric ion in a nearly identical fashion (Bruns *et al.* 1997). It is currently unknown whether FbpA contributes to the iron removal and transport process or whether merely captures iron released into the periplasmic space. In the present study, site directed mutants of FbpA were prepared so that the role of FbpA in the iron uptake from lactoferrin could be assessed through gene replacement strategies.

Materials and methods

Construction of site-directed FbpA mutants

The various site-directed mutants of the *H. influenzae* fbpA gene were prepared either by the Stratagene QuikChange Site-Directed Mutagenesis kit or by a two-step PCR mutagenesis protocol using the pT7-7 plasmid encoding the wild-type gene as the starting template. Sequence analysis confirmed that the desired mutations were the only changes in the coding sequence. A gene replacement vector for *Neisseria meningitidis* was prepared by cloning a 500 bp of the region immediately upstream of the *fbpA* gene adjacent to a 1.3 kb region containing the *fbpAB* intergenic region and a portion of the coding sequence for the *fbpB* gene. Restriction sites engineered between these two regions were used to clone in a chloramphenicol resistance cassette derived from the pTnMax4 plasmid (Haas *et al.* 1993) or the kanamycin resistance determinant derived from the pUC4K plasmid. The plasmid containing the chloramphenicol resistance cassette was linearized by digestion with *Sst1* and used directly to transform *N. meningitidis* strain B16B6. The resulting chloramphenicol resistant strain was used as a parent for all subsequent transformations. The wild-type or mutant *H. influenzae fbpA* genes were subcloned from the pT7-7 vector into

the gene replacement vector containing the kanamycin resistance cassette between the upstream region and kanamycin resistance determinant. The resulting plasmids were linearized by *Sst1* digestion and used to directly transform the chloramphenicol resistant parent strain.

Purification and crystallization conditions of Q58L PbpA

E. coli BL21(DE3)/pLysS containing the pT7-7 plasmid encoding the Q58L mutant FbpA was utilized to express protein in the periplasm under isopropyl-1-thio-β-D-galactopyranoside induction. Purification and crystallization were performed as previously described (Shouldice *et al.* 2003a) with the following exceptations. Crystals of Q58L were grown at 4 °C by the hanging drop technique from 4 μL drops containing 15 mg/mL of the Q58L FbpA, 18% PEG 550 MME, and 0.05 M Tris pH 8.5. The drops were equilibrated against a 1 mL reservoir containing 36% PEG 550 MME, and 0.1 M Tris pH 8.5. Diffraction quality pink crystals typically grew after four days. For cryocrystallography, the crystals were placed in a cryoprotectant solution identical to the reservoir solution with a final concentration of 20% (v/v) ethylene glycol for a short time before being placed in the nitrogen gas stream to be cooled to 100 K.

Data measurement and structure solution

Initial X-ray analysis revealed that all crystals belong to the orthorhombic space group $P2_12_12$ with unit cell dimensions of $a = 105.77$ Å, $b = 75.74$ Å, $c = 33.95$ Å, $\alpha\beta\gamma = 90°$. Crystallographic data were recorded utilizing the same equipment as the data for the H9A mutant previously described (Shouldice *et al.* 2003b). The steps taken and programs used in determining the 1.7 Å resolution Q58L structure were identical to those utilized in solving the previous H9A crystal structure (Shouldice *et al.* 2003b). The data was 89% complete with R_{free} value of 24.8 and an R_{factor} of 19.5. Ramachandran plot of the mutant structure reveal that 92.4% of the residues are in the most favored regions and 7.6% fall in additional allowed regions indicating the Q58L structure is of high quality.

Growth studies

A screening growth assay was performed using the original B16B6 strain (N16) as a positive control and the FbpA-ve, chloraphenicol resistant parent strain (N322) as a negative control. Freshly isolated colonies from BHI plates were used to inoculate starter cultures of BHI broth buffered to pH 7.4 with 50 mM HEPES. Late log phase cultures (approximately 6 h of growth) were subcultured at a 1 in 100 dilution into 2 mls of buffered BHI broth containing 100 uM EDDHA and 0.2 mg of iron-loaded hTf or hLf. Cultures were grown overnight and observed for up to 48 hrs. A final A_{600} of less than 0.1 was considered negative whereas positive cultures had a final A_{600} of greater than 0.9.

Table 1. Site-directed mutants of the *Haemophilus influenzae* FbpA

Residue	Binds to	Mutated to	Name
His 9	Fe	Ala	H9A
		Gln	H9Q
Glu 57	Fe	Ala	E57A
Gln 58	PO4	Leu	Q58L
Asn 175	PO4	Leu	N175L
Asn 193	PO4	Leu	N193L
Tyr 195	Fe	Ala	Y195A
Tyr 196	Fe	Ala	Y196A
Gln58/N175	PO4	Leu	double
Gln58/N175,N193	PO4	Leu	triple

Results

Construction and characterization of site-directed FbpA mutants

The overall strategy for evaluating the role of iron binding by the periplasmic protein in the transport process was to prepare a series of site-directed mutants and evaluate growth on lactoferrin (or transferrin) after replacing the wild-type FbpA protein. This strategy also formed the basis of a complementary study (Khan *et al.* 2003) that evaluated the role of FbpA on transport from transferrin in the human pathogen, *H. influenzae*. The absence of a lactoferrin receptor-mediated iron acquisition pathway in *H. influenzae* precluded us from evaluating uptake from lactoferrin in this species, and prompted us to attempt to examine this pathway in *N. meningitidis* in this study.

The four amino acids involved in directly coordinating the iron atom and the three amino acids involved in binding the coordinating phosphate anion were

targeted for site-directed mutagenesis (Table 1). Site-directed mutants of the *H. influenzae* FbpA gene readily provided substantial quantities of stable protein in the periplasm of *Escherichia coli* that were suitable for biochemical and structural analysis. In contrast to the preparation of wild-type FbpA, which possessed a strong red coloration, all of the mutant preparations were either colorless or had a substantially reduced coloration. Although this suggested a relative deficiency in iron binding of the mutant proteins, all of the mutant proteins could be fully iron-loaded using our standard methods (data not shown).

Concentrated preparations of the iron loaded protein samples were used to grow crystals by vapor diffusion using the hanging drop technique. The conditions that were successful for the wild-type protein (Bruns *et al.* 1997) served as a starting point for crystallization screens. Diffraction quality crystals of the Glu58Leu (Q58L) mutant protein obtained after four days growth under optimal buffer conditions were placed in a cryoprotectant solution, cooled and subjected to X-ray diffraction analysis. A model for the Q58L protein was derived from the processed data by molecular replacement using the structure of the wild-type apo protein (1D9V) as the initial phasing model.

There was substantial separation between the N-terminal and C-terminal domains of the iron-loaded Q58L mutant protein such that the alpha carbon chain could be superimposed on the apo form of the wild-type protein (Figure 1A). The other mutant FbpAs were also in the open conformation in the crystallized state (data not shown), in contrast to the iron-bound form of the wild-type protein (Figure 1A). In spite of crystallizing in the open conformation, it is likely that mutant FbpA proteins such as Q58L fluctuate between the open and closed conformation in solution. The presence of a large EDTA molecule (Shouldice *et al.* 2003a) or iron-hydroxide clusters (Shouldice *et al.* 2003b) in some of the mutant FbpAs could impair adoption of the closed conformation.

The two tyrosine hydroxyls (Y195 and Y196) in the C-lobe of the Q58L mutant protein were involved in iron coordination, but the imidazole nitrogen from His 9 and the carboxylate oxygen of Glu 57 from the N-Lobe were too distant to be involved. There was a bound phosphate molecule (Figure 1B) in the same position as in the wild-type iron-loaded and apo proteins, providing a third coordinating ligand for the iron atom. The remaining coordinating ligands appear to be provided by bound water molecules. Displacement of two bound water molecules by His 9 and Glu 57 upon domain closure would provide the same coordinating atoms as in the wild-type protein. Thus one would anticipate that the iron-binding properties of the Gln58 mutant protein would not be severely impaired.

Gln 58 is involved in coordinating the bound phosphate anion in the wild-type, iron-loaded protein but clearly is not essential since there also is a bound phosphate in the apo form of the protein. Clearly the liganding residues from the C-lobe (i.e. Asn175, Asn193) are sufficient for coordinating the phosphate anion. Thus the only deficiency in iron and anion coordination in the Q58L mutant protein would be the absence of the Gln nitrogen for completing the coordination of the phosphate anion in the closed conformation. This suggests that there would be a very moderate effect on the anion and iron binding by the Q58L FbpA.

Preparation of mutant strains

The strategy for preparation of mutant strains was to take advantage of the natural transformation system present in *N. meningitidis* and use linear DNA to effect the desired gene replacements. As a first step, we decided to replace the native *N. meningitidis fbpA* gene with an antibiotic resistance cassette so that we would eliminate unwanted crossovers between the mutant *fbpA* genes being introduced and the resident *N. meningitidis fbpA* gene (Figure 2). We cloned a segment containing the promoter and upstream region from the *fbp* locus (but lacking the ribosomal binding site) adjacent to a fragment containing the *fbpA-B* intergenic region and a portion of the *fbpB* gene in the pBluescript vector with suitable restriction sites between the two flanking regions. After cloning a chloramphenicol resistance cassette in between the flanking regions, the linearized vector was used to transform *N. meningiditis* strain B16B6 and the transformed cells were plated onto plates containing chloramphenicol. A relatively small number of chloramphenicol resistant colonies were obtained. The colonies were screened for growth in the presence of transferrin and lactoferrin and for the loss of the native *fbpA* gene by PCR analysis. An isolate with the appropriate characteristics, N322, was selected as the parent strain for subsequent gene replacements. This strain had the advantageous feature of being highly transformable, yielding 2–3 orders of magnitude more transformants from a single transformation reaction.

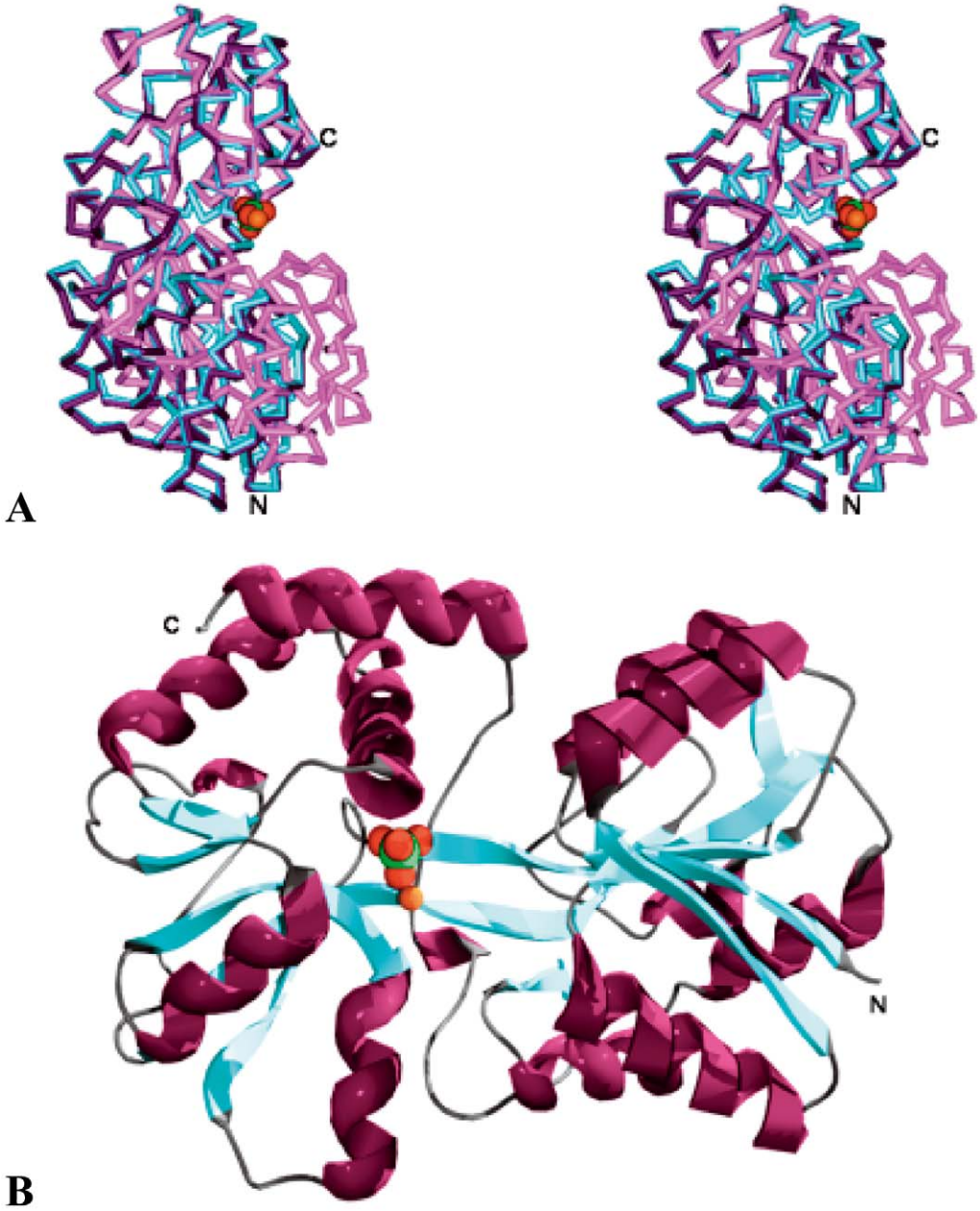

Fig. 1. Stereo Cα plots of *Haemophilus influenzae* FbpA structure (A) and ribbon diagram of Q58L structure (B). (A) The apo (purple) and holo (pink) forms of the wild type protein are superimposed with the Q58L structure (cyan). The model were superimposed on the C-terminal domain. The holo and apo structures were derived from their respective PDB files (1MRP and 1D9V). The phosphate molecule and iron atom (orange) contained in the holo form are shown. (B) Identical to the wild type structures, Q58L FbpA possesse two α/β domains linked by two antiparallel β-strands. α-helices are displayed in purple, while β-strands are shown in cyan. The relative binding modes of the phosphate anion and iron atom (orange sphere) are also shown.

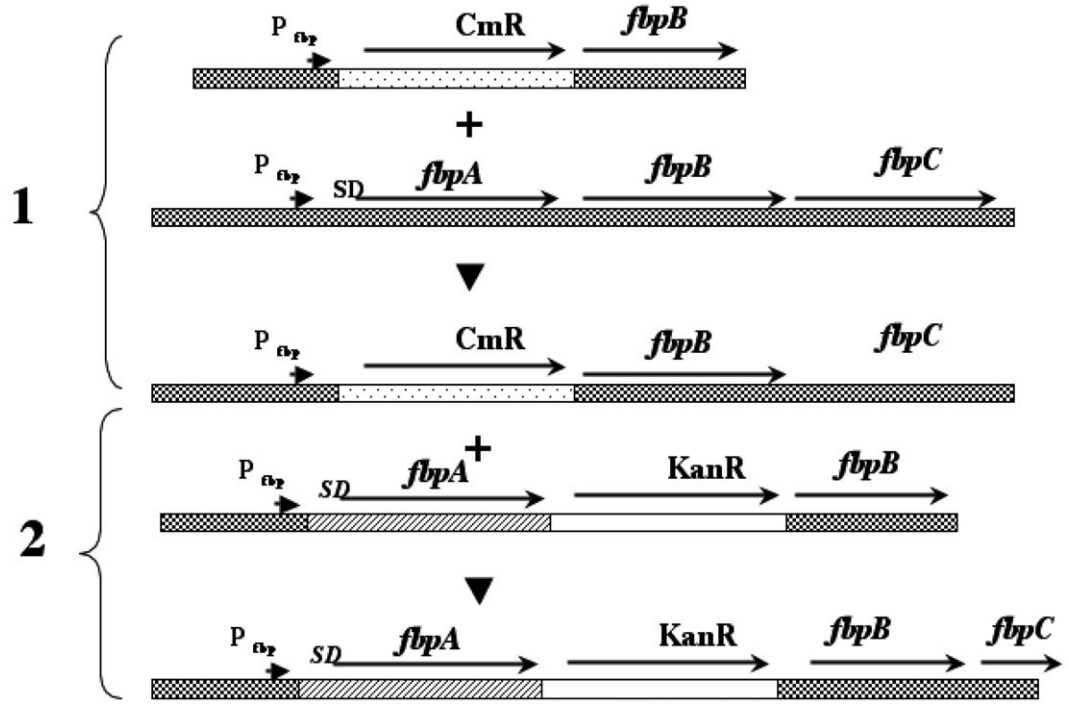

Fig. 2. Gene replacement strategy. Step 1. A chloramphenicol resistance casette cloned between the upstream region and partial *fbpB* gene was used to transform the wild-type locus, generating a chloramphenicol resistant strain. Step 2. A 'foreign' *fbpA* gene (with upstream SD) cloned between the fbp promotor/upstream region and a kanamycin resistance casette followed by the partial *fbpB* gene was used to transform the chloramphenicol resistance strain.

Initially we decided to transfer several of the mutant *H. influenzae fbpA* genes into *N. meningitidis* based on preliminary results from our laboratory demonstrating that the *H. influenzae* FbpA could functionally replace the *N. meningitidis* FbpA in a reconstituted FbpABC pathway in *E. coli*. To provide a selectable marker for cloning the mutant *fbpA* genes into *N. meningitidis*, a kanamycin resistance determinant was introduced between the flanking regions leaving convenient restriction sites for directional cloning of the foreign *fbpA* genes. Subsequent cloning of the *fbpA* genes from *H. influenzae* would result in the kanamycin resistance determinant being positioned between the foreign *fbpA* gene and the *fbpAB* intergenic region with the expectation that it should not substantially alter the expression of the native *fbpB* and *fbpC* genes. The wild-type and mutant *fbpA* genes were ligated with the digested vector containing the kanamycin resistance cassette and plasmids containing the hybrid *fbp* operons were isolated from *E. coli* and the insertion of *fbpA* in the correct orientation was determined by PCR and/or restriction digests of the hybrid operons. Plasmids containing the wild-type or mutant *fbpA* genes were linearized with *Sst*I and used to transform *N. meningitidis* strain N322, selecting for kanamycin resistant colonies (Figure 2). Colony PCR analyses were performed to ensure that an intact *fbpA* gene was present in the genomic DNA of the resulting transformants.

Table 2. Growth on various iron sources

Strain	FbpA Type	FbpA Species	Growth of source of Iron	
			HTf	HLf
N16	Wt	*N. meningitidis*	++	++
N322	None		–	–
N326	Wt	*H. influenzae*	–	–
N323	H9Q	*H. influenzae*	–	–
N324	Q58L	*H. influenzae*	–	–
N325	triple	*H. influenzae*	–	–

Growth studies

The series of strains containing mutant *H. influenzae fbpA* genes were tested for growth on iron-deficient medium supplemented with iron-loaded hTf of hLf to evaluate their ability to mediate transport of iron from hTf or hLf (Table 2). To account for the effects of using a foreign FbpA and of inserting the kanamycin resistance gene upstream of the *fbpB* and *fbpC* genes, a control with the wild-type *H. influenzae* FbpA was included in this experiment. Unfortunately, the strain containing the wild-type FbpA gene did not support growth on Tf or Lf, which prevented us from evaluating the effect of the specific mutations on the iron acquisition process. Thus these results suggest that there may be a barrier to functional expression of the *H. influenzae* FbpA that might be due to the presence of a foreign signal peptide and/or ribosomal binding site. A set of mutant *N. meningitidis fbpA* genes that are currently being prepared will be essential for determining the effect of the specific mutations on iron acquisition from hLf and hTf.

Discussion

Members of the family Neisseriaceae are Gram-negative bacteria that are highly adapted to living in the environment of the host. The human pathogens *N. meningitidis, Neisseria gonorrhoeae*, and *Moraxella catarrhalis* primarily inhabit mucosal surfaces in the human respiratory or genital tract and are only rarely present within the body during invasive disease. When grown in iron-limited media *in vitro* they are capable of using exogenous human transferrin or lactoferrin as the sole source of iron for growth (Campagnari *et al.* 1994, Mickelsen & Sparling 1981, Mickelsen *et al.* 1982). Specific surface receptor proteins that bind the host glycoproteins are required for the process of iron acquisition since insertional mutagenesis of the receptor protein genes abrogates use of these proteins as an iron source (Biswas & Sparling 1995, Bonnah & Schryvers 1998, Bonnah *et al.* 1999, Cornelissen *et al.* 1992, Du *et al.* 1998, Irwin *et al.* 1993). Studies with a human model of *N. gonorrhoeae* infection demonstrated that human transferrin can serve as a iron source on mucosal surfaces *in vivo* since loss of the transferrin receptor (from a strain naturally deficient in the lactoferrin receptor) impaired growth and survival of the bacteria (Cornelissen *et al.* 1998). A more recent study using a strain with a functional lactoferrin receptor and lacking a transferrin receptor, demonstrated that lactoferrin can also serve as an effective iron source *in vivo* (Anderson *et al.* 2003). In addition, a competition experiment with two infecting strains demonstrated that the presence of a lactoferrin receptor conferred a selective advantage even in the presence of a transferrin receptor (Anderson *et al.* 2003).

The lactoferrin receptor is composed of two proteins, lactoferrin binding protein B and A (LbpB and LbpA), encoded by the *lbpB* and *lbpA* genes (Du *et al.* 1998, Lewis *et al.* 1998, Pettersson *et al.* 1998). However, the ability to bind to human lactoferrin has not been consistently demonstrated for LbpB in solid-phase binding assays and affinity isolation experiments, and its role in iron acquisition has not been experimentally established, as isogenic *lbpB* deficient mutants in pathogenic *Neisseria* species or *Moraxella catarrhalis* are not defective in lactoferrin-dependent growth or iron acquisition. Clearly further studies will be required to establish a role for this protein.

LbpA-ve isogenic mutants from *N. meningitidis, N. gonorrhoeae* and *M. catarrhalis* (Biswas & Sparling 1995, Bonnah *et al.* 1999, Quinn *et al.* 1994) were completely defective in growth with lactoferrin as an iron source. These results indicate that LbpA is capable of mediating the iron removal and uptake process, in conjunction with the other pathway components such as FbpA (Khun *et al.* 1998) and TonB (Stojiljkovic & Srinivasan 1997). Although the participants in the iron removal and uptake process are known, the mechanism has not been determined. Our current model for this process proposes that conformational changes occur in lactoferrin upon receptor binding that results in domain separation and lowers the affinity of receptor-bound lactoferrin for iron (Figure 3). We also propose that the high affinity iron binding by FbpA provides a driving force for transport of iron across the outer membrane once a channel has been provided through interaction of LbpA with TonB. The complexity of this uptake system presents a barrier to developing reconstituted systems for directly addressing mechanistic features. However, there are a couple of predictions derived from our model that can be tested experimentally. The first prediction is that LbpA should interact with both domains of a lobe to effect domain separation and the second prediction is that FbpA variants with lower iron binding capabilities should be defective in the iron removal and transport process.

A recent study involving the use of protein hybrids between human lactoferrin and bovine transferrin has

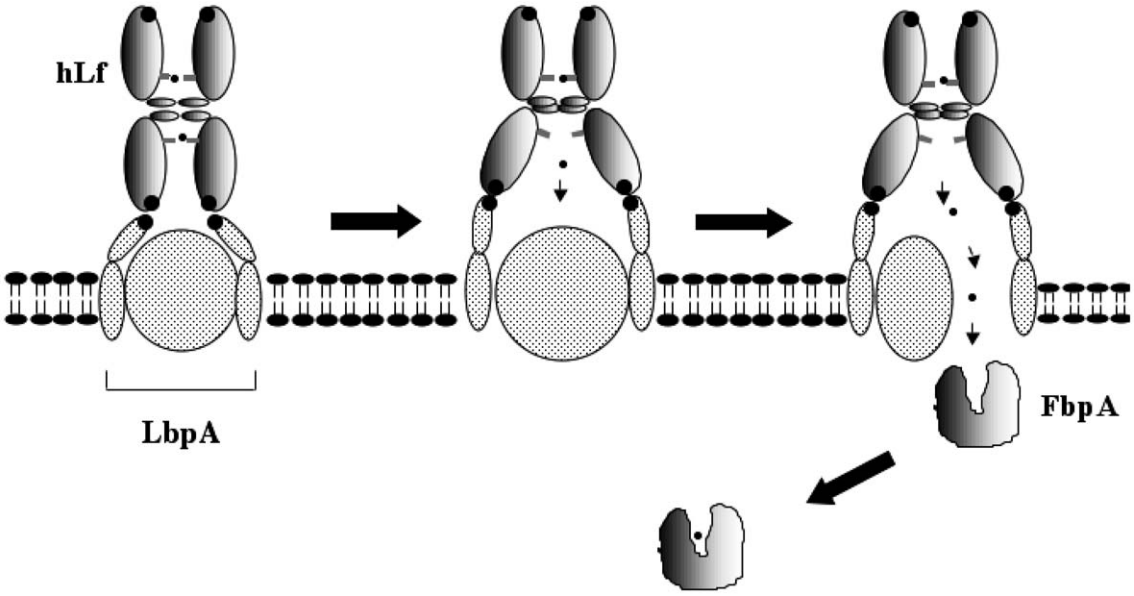

Fig. 3. Model for transport of iron from lactoferrin across the outer membrane. Iron-loaded hLf binds to the surface receptor, LbpA, involving binding sites on both domains of the C-lobe. The receptor-ligand interaction induces conformational changes which result in lowering of the binding affinity of the C-lobe for iron. The interaction of LbpA with TonB (not shown) provides a channel for transport of iron across the outer membrane.

provided envidence that LbpA recognizes both domains in the C-lobe of human lactoferrin (Wong & Schryvers 2003). The sites recognized by LbpA were not precisely identified in this study and neither were the regions on LbpA mediating these interactions. Additional studies delineating the sites of interaction on lactoferrin and LbpA could provide further insights into the iron removal process. Nevertheless, the current findings are consistent with the proposed model of iron removal and uptake (Figure 3).

To address the second component of our model for iron transport, we prepared a set of site-directed mutants of the *H. influenzae* FbpA to be used for replacement of the native protein. The mutant proteins have been stably produced in the *E. coli* periplasm and the iron-loaded forms of these proteins were characterized by structural analysis (Shouldice *et al.* 2003a, Shouldice *et al.* 2003b) (Figure 1). Replacement of the native FbpA in *H. influenzae* with a number of these mutant proteins results in a loss in ability to use transferrin as a source of iron for growth (Khan *et al.* 2003), which supports our model of iron acquisition. To evaluate the role of iron-binding by FbpA in iron acquisition from lactoferrin, we introduced a subset of the mutant protein genes into *N. meningitidis*. Unfortunately the control wild-type *H. influenzae* protein was not capable of supporting growth, suggesting that there may be a barrier to functional expression of the *H. influenzae* FbpA. Thus we are currently preparing a set of site-directed mutants of the *N. meningitidis fbpA* gene to address this issue. Further studies using this overall approach may enable us to provide an estimate of the binding constant of the lactoferrin-receptor complex *in vivo* and evaluate the contribution of individual components, such as LbpB, or regions of components (regions of LbpA) on the iron removal process.

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