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THE ROLE OF IRON-BINDING PROTEINS IN THE SURVIVAL OF PATHOGENIC BACTERIA[1]

Timothy A. Mietzner
Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Stephen A. Morse
Division of Sexually Transmitted Disease Laboratory Research, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333

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THE ROLE OF IRON-BINDING PROTEINS IN THE PATHOGENESIS OF INFECTIOUS DISEASE

The process of infectious disease as it relates to pathogenic microorganisms depends largely on the efficiency with which these organisms (*a*) gain access to the host environment, (*b*) colonize, (*c*) cause pathology, and (*d*) disseminate

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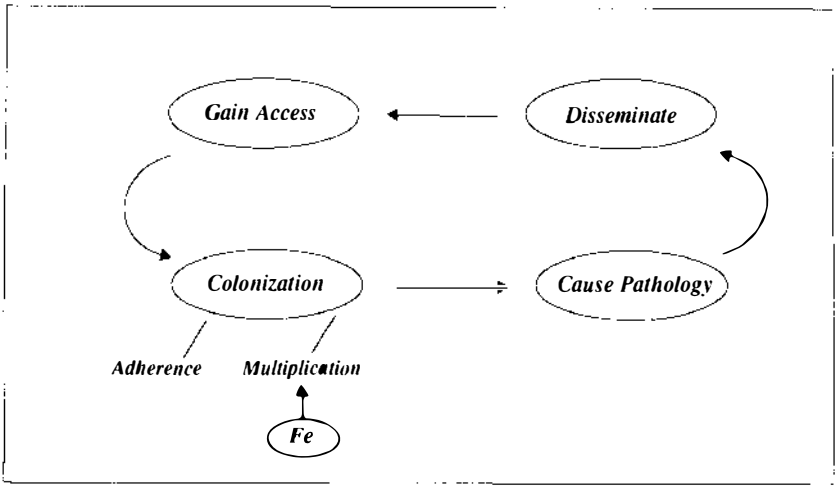

Figure 1 General steps in the infectious cycle indicating where the role of iron is relevant.

to a new host. This process is referred to as the pathogenic cycle (Figure 1), and for many pathogenic microorganisms, its efficient iteration is critical for survival. The role of iron-binding proteins in this cycle is most prominent in the process of colonization, which involves two discrete steps—adherence and multiplication. Adherence allows establishment of infection at discrete sites, whereas multiplication allows pathogenic microorganisms to reach a critical mass within this microenvironment. The process of multiplication requires acquisition of growth-essential nutrients, including iron, from the host. All pathogenic bacteria require iron as a growth-essential nutrient. Little free iron is found in the human host because most is complexed to proteins, which represent the principal reservoir of growth-essential iron for pathogenic bacteria (109, 110). Therefore, the ability of pathogenic microorganisms to scavenge iron from their host environment and incorporate this element into proteins is a fundamental requirement for the production of disease. For the purposes of this review, an iron-binding protein is defined as one that binds iron directly or that binds iron/protein ternary complexes. Several excellent reviews on aspects directly or peripherally related to this topic have been published previously (e.g. 12, 22–24, 32–35, 52, 74–77, 87, 88, 101, 105–112). We refer interested readers to these sources.

Chemistry and Biologic Distribution of Host Iron

On a molar basis, hydrogen, carbon, and nitrogen are the predominant elements that compose living cells. Iron is required in small quantities that generally

constitute the reaction centers of many protein-mediated processes. Iron is the fourth most abundant element in the Earth's crust and would not appear to limit biologic growth. However, under physiologic conditions, iron exists in the ferric state and is only sparingly soluble (109). This insolubility would not pose a significant problem if free iron were only required as a trace element. However, at 0.005% (w/w), iron ranks fifth on a per weight basis (behind calcium, phosphorous, sodium, and magnesium) as an inorganic constituent of a 70 kg adult male. This percentage represents a 20-fold excess over copper, the next most abundant element, and a 50-fold excess over zinc (109).

Vertebrates control acquisition of iron by tightly regulating the amount absorbed and excreted. In humans, 5~15% of orally ingested dietary iron (~ 1 mg/day) is normally absorbed into the internal iron pool. From this pool of iron, ~ 1 mg/day is excreted (109), which balances intake with output. Most of the absorbed iron is confined to intracellular compartments where it either serves as an essential cofactor for several diverse biologic processes or is stored in the form of iron-ferritin complexes from which iron can be mobilized upon cellular demand. Although all cells in the human body require iron, the majority is stored in developing and mature red blood cells or in cells of the liver, spleen, and muscle (109). Red blood cells contain ~ 60% of body iron, and the concentration of intraerythrocytic heme iron can approach 20 mM. The spleen and liver are the two principal sites of iron storage, accounting for ~ 25% of body iron. Muscle sequesters some 11% of total body iron, and iron in plasma and secretions accounts for 4% (109).

Iron does not have a single biologic function but rather is a cofactor in a wide variety of chemical reactions (109). Although this versatility explains why iron is so important to living cells, it is nonetheless inconsistent with its insolubility. On the other hand, free iron is toxic—even at low concentrations—as a result of Fenton chemistry, which involves iron-catalyzed production of toxic hydroxyl radicals (109). Nature has "brokered" these opposing properties by allowing coordination of free iron with a diverse array of compounds such as salts, organic acids, and proteins. Iron-binding proteins apparently solubilize iron at physiologically relevant concentrations and limit its toxicity by providing a "suitably tailored protein environment" (109) in which it can act as a catalyst for biologic reactions.

HOST IRON-BINDING PROTEINS: THE RESERVOIR OF IRON FOR MICROBIAL PATHOGENS

General Classes of Host Iron-Binding Proteins

Iron coordinates with proteins in both the Fe^{3+} and the Fe^{2+} state. In the Fe^{3+} state, six coordination sites are available for binding vs four in the Fe^{2+} state.

Iron may be bound to protein through either all or a subset of these sites. An additional compound, such as protoporphyrin or O_2, may also be involved. Iron-binding proteins generally perform a critical function in metabolism (e.g. electron transport, oxygen transport) or in the transport and storage of iron. In either case, how iron is bound clearly dictates how it is utilized, providing a rationale for dividing the human iron-binding proteins into five major categories based on the nature of the bound iron. These categories are (*a*) iron-transport proteins (siderophilins), (*b*) iron-storage proteins, (*c*) heme/protein complexes, (*d*) iron-sulfur proteins, and (*e*) nonheme, nonsulfur iron proteins.

IRON-TRANSPORT PROTEINS Iron-transport proteins share a common iron-binding motif and are referred to as siderophilins (88) to underscore that this group represents a superfamily of iron-binding proteins that possess a high affinity ($> 1 \times 10^{20}$) for Fe^{3+}. Siderophilins are glycoproteins with molecular masses of ~ 80,000 that bind two molecules of Fe^{3+} per molecule of protein. Two siderophilins important in the pathogenesis of infectious diseases are serum transferrin and mucosal lactoferrin. Each of these proteins is comprised of ~ 700 residues sharing 35–40% identical residues when optimally aligned (32). X-ray crystallographic analysis of human lactoferrin reveals that it consists of two lobes, each of which binds a single molecule of iron. Each lobe is comprised of two domains with a characteristic alternating α-helix/β-sheet folding motif (1, 8). The iron-binding site lies within the cleft between these domains. Each siderophilin lobe binds a single molecule of Fe^{3+} through two tyrosines, one histidine, one aspartate, and a carbonate anion. This coordination profile is remarkably well conserved among the siderophilins (22, 32). In addition, binding is not restricted to Fe^{3+}; other metals (e.g. Ga^{3+}, Cu^{2+}, Al^{3+}, Zn^{2+}) can be bound as well, albeit at lower affinities than iron (22).

Transferrin, the predominant human siderophilin, is a 679-residue glycoprotein found in plasma and lymphatic fluids at concentrations of ~ 2.3 mg/ml (30 μM) (110). It is also found in tears, cerebrospinal fluid, bile, amniotic fluid, milk, saliva, aqueous humor, and seminal fluid at concentrations of one to two orders of magnitude less than in serum (110). Transferrin is involved in the extracellular transport of iron between cells. It is synthesized in the liver and is typically 33% iron saturated in healthy adults. Similar to transferrin, lactoferrin is a 703-residue glycoprotein that binds iron with high affinity (32). Unlike transferrin, it is found predominantly in mucosal secretions (62) and phagocytic granules (110) and is usually less than 10% iron saturated (18). At neutral pH, lactoferrin has a 300-fold greater affinity for iron than does transferrin. At decreased pH (~ 4.5) lactoferrin retains its high affinity for iron, whereas iron can be mobilized easily from transferrin (87, 110). The primary function of lactoferrin appears to be inhibition of microbial growth; it is not considered a principal participant in eukaryotic iron transport. The concentra-

tion of lactoferrin in serum is less than 0.3 μM (110); iron-saturated lactoferrin is rapidly removed from circulation (87).

Lactoferrin is thought to be bacteriostatic for microorganisms by virtue of its ability to sequester iron at high affinity (11). However, Arnold et al reported that iron-saturated lactoferrin is bactericidal, particularly against the gram-positive dental pathogen *Streptococcus mutans* and the gram-negative enteric pathogen *Vibrio cholerae* (11). Several studies have indicated that lactoferrin binds to outer membrane proteins or to lipopolysaccharide of gram-negative bacteria, which would position it to exert its bactericidal function (71, 89b, 93). Ambruso & Johnson suggested that the local formation of toxic hydroxyl radicals by the iron bound to lactoferrin is responsible for this bactericidal activity (6). Alternatively, another report indicates that a peptide derived from the N-terminal region of lactoferrin was bactericidal owing to its ability to perturb membranes (115). A clear mechanism(s) for lactoferrin inhibition of pathogenic microorganisms remains to be established.

Transferrin appears to be the primary siderophilin mediating bacteriostasis in serum because its concentration is 100-fold greater than that of lactoferrin. However, the concentration of lactoferrin can increase ~ 60-fold in response to infection. Moreover, lactoferrin may play a prominent secondary role in maintaining the sterile environment of the serum (87). The largest concentrations of lactoferrin are found at sites of potential microbial invasion (mucosal secretions, tears, etc). Lactoferrin likely limits the growth of organisms on nonsterile mucosal surfaces and is also involved in inhibiting growth of intracellular organisms (87). However, the contrary may also be true. Byrd & Horowitz reported that exogenous transferrin- or lactoferrin-bound iron can reverse the γ-interferon-dependent inhibition of *Legionella pneumophila* intracellular multiplication (25).

IRON-STORAGE PROTEINS Ferritins are involved in the storage and detoxification of intracellular iron. These iron-binding proteins are unique because they do not specifically bind to an individual iron atom but rather to an iron-salt aggregate (52). Ferritins are organized as soluble protein shells (12–13 nm in diameter) with a spheric (7–8 nm in diameter) core. *Apo*-ferritin is comprised of two subunits, H (22–24 kDa) and L (20–22 kDa). These are arranged in a heterogeneous complex of 24 subunits ranging from all H to all L and all combinations in between. *Apo*-ferritin catalyzes the oxidation of ferrous iron to ferric iron. The iron-rich core of mammalian ferritin consists of up to 4500 atoms of iron in the form of insoluble ferrihydrite ($5Fe_2O_3 \cdot 9H_2O$) (52). Ferritin is found in greatest concentration in the spleen, liver, and bone marrow (109). In addition to storing iron, ferritin may play a role in sequestering free iron during periods of iron overload. In this capacity, it would not be used for storage per se but for the detoxification of free iron within a cell (101). Bacteria

also produce ferritin analogs, referred to as bacterioferritins, that are involved in the storage of iron and perhaps in the detoxification of intracellular iron (52, 101).

HEME/PROTEIN COMPLEXES Heme/protein complexes consist of an iron porphyrin complexed to a protein subunit. Porphyrins are cyclic compounds formed by the linkage of four pyrrole rings (protoporphyrin) that complex with iron through a nitrogen atom contributed by each of the rings. The fifth iron coordination site is associated with the protein subunit, often through the imidazole ring of a histidine residue. The sixth coordination site often determines the function of the protein. In the case of oxygen-carrying proteins such as hemoglobin or myoglobin, the natural ligand is oxygen. In other cases the sixth coordination site is the protein molecule itself wherein iron participates in redox reactions for the purpose of electron transport (e.g. as with cytochromes). Other members of this class of proteins include catalase and peroxidase, which break down toxic hydrogen peroxide.

Hemoglobin and myoglobin are normally intracellular proteins. However, upon tissue destruction, which may occur during bacterial infection, they can be released into extracellular fluids such as plasma. Haptoglobin is present in plasma at a concentration of 1 mg/ml (1.2 μM). It specifically and irreversibly binds hemoglobin and its derivatives (63). Haptoglobin is synthesized within the liver and has a half-life of ~ five days when it is not complexed to hemoglobin. However, when complexed with hemoglobin, haptoglobin has a half-life of ~ 15 min (53). Haptoglobin probably prevents renal loss of hemoglobin and sequesters hemoglobin-bound iron from microbial pathogens.

For all practical purposes, free heme is insoluble under physiologic conditions and can only be maintained in a soluble form by binding to hemopexin or albumin. Hemopexin is an extensively glycosylated protein with a molecular mass of ~ 60,000. It is synthesized in the liver and is found in serum at concentrations of ~ 0.75 mg/l (1.1 μM) (73). Each hemopexin molecule can bind one molecule of heme with a $K_{diss} = 10^{-13}$. *Apo*-hemopexin has a half-life of ~ seven days, whereas the hemopexin-heme complex is removed from circulation by hepatic parenchyma cells with a half-life of 7–8 h. Albumin binds heme at a 1:1 molar ratio to form a methemalbumin complex. The affinity of albumin for heme is substantially lower than that of hemopexin, with a $K_{diss} = 10^{-8}$ (49).

IRON-SULFUR PROTEINS Iron-sulfur proteins represent a large group of proteins with a wide variety of activities (109). However, they are generally present at limiting concentrations, depending on the cell type, and probably do not represent a major source for binding by microbial iron-containing proteins. In their simplest form, these proteins complex iron through cysteinyl

residues (109). More complex iron-sulfur proteins incorporate inorganic sulfur as part of the coordination site. Examples of this class of proteins include adrenodoxin, which participates in the hydroxylation of steroid hormones and vitamin D; ferrodoxin-like proteins associated with mitochondrial respiratory complexes; xanthine oxidase, an enzyme that catalyzes the hydroxylation of purines; aconitase, a mitochondrial enzyme of the Krebs cycle; and amido phosphoribosyl transferase, an enzyme that catalyzes the first reaction in the de novo synthesis of purines (109).

NONHEME, NONSULFUR IRON PROTEINS Nonheme, nonsulfur iron proteins can be subdivided into the iron oxygenases and the binuclear *oxo*-bridged iron proteins. Like iron-sulfur proteins, these iron-binding proteins probably are not an important source of iron for invading microbial pathogens. Mono-oxygenases (e.g. phenylalanine hydroxylase) catalyze hydroxylation reactions in which one oxygen atom is attached to the substrate while the other atom combines with hydrogen to form water. Dioxygenases (e.g. proline hydroxylase) catalyze the introduction of both atoms of molecular oxygen into a substrate. Other binuclear *oxo*-bridged iron proteins include ribonucleotide reductase, an essential enzyme that catalyzes the conversion of ribonucleotide diphosphates into deoxyribonucleotides, and uteroferrin, a mammalian enzyme the catalyzes the removal of protein-bound phosphates (109).

IRON-BINDING PROTEINS OF PATHOGENIC BACTERIA

Iron-Binding Proteins and Metabolism of Pathogens

For the most part, the roles of iron-containing proteins in pathogens are analogous to those in the host. Many of these proteins are essential enzymes required for the growth and survival of pathogenic microorganisms. They may also be virulence factors. For example, a common defense mechanism of human neutrophils is the production of toxic oxygen radicals. Pyogenic staphylococci, which are extracellular pathogens, produce large quantities of the iron-binding protein catalase to limit the oxygen-dependent killing by neutrophils. Gonococci and *Helicobacter pylori* also produce large quantities of catalase (76, 77), but naturally occurring catalase-deficient organisms can be isolated from infected individuals (12, 34). In contrast to staphylococci, these organisms are facultative intracellular pathogens (i.e. they are internalized by epithelial cells as part of the infectious process). Because the potency of oxidative killing by professional phagocytes (e.g. neutrophils) relative to non-professional phagocytic cells (e.g. epithelial cells) is greater, one can infer that catalase production may not play the pivotal role in helicobacter- and gonococcal-associated infection that it does in staphylococcal infection.

Recently, Mengaud & Horwitz identified several iron-containing proteins of *L. pneumophila* (22). The major iron-containing protein (MICP) had a molecular mass of 210 kDa and shared sequence homology with *Escherichia coli cis*-aconitase. Under identical conditions, the majority of *E. coli* iron was associated with a 450-kDa protein presumed to be bacterioferritin. It was estimated that 100,000 copies of MICP were present in each *L. pneumophila* cell, a very high number comparable to the number of porins within the gram-negative cell envelope. Whereas most pathogenic bacteria require 0.3–1.6 μM iron for growth, *L. pneumophila* requires >20 μM (22). The abundance of MICP suggests that *L. pneumophila* has a high metabolic requirement for aconitase, which in turn may explain the high iron requirement of this pathogenic microorganism.

Iron-Binding Proteins and Iron Acquisition by Pathogenic Microorganisms

The high-affinity acquisition of iron from the host environment is a necessary but perhaps insufficient determinant of virulence. High-affinity iron-acquisition systems require proteins that directly associate with iron during the iron recruitment, transport, and storage processes. The current literature divides these systems into two categories: those mediated by a conventional siderophore and those mediated by siderophilin-binding processes (described below).

SIDEROPHORE-MEDIATED IRON ACQUISITION The classical definition of a siderophore is a nonporphyrin, nonprotein, iron-binding compound of low molecular weight that has a high formation constant for iron, is repressed in the presence of iron, and can act as the sole source of iron for the species producing it as well as for other coexisting microbial species (24). Most siderophores fall into two chemical classes, phenolates (catechols) and hydroxamates (33). Within these classes, the chemical structures of siderophores are diverse. The prototypical phenolate siderophore is enterochelin, a cyclic trimer of 2,3-dihydroxy-N-benzoyl-L-serine with a K_{ass} for Fe^{3+} of 1×10^{37} that is produced by many gram-negative pathogens. Aerobactin is prototypical of the hydroxamate class of siderophores. It is a conjugate of 6-(N-acetyl-N-hydroxyamino)-2-aminohexanoic acid and citric acid (35) and is the only known hydroxamate siderophore produced by *E. coli*. The synthesis of aerobactin can be directed by the ColV-K30 plasmid associated with invasive strains of *E. coli* (35). Other studies demonstrate that the chromosome can also encode aerobactin synthesis genes. In one study, >50% of human isolates from feces and blood produced a hydroxamate siderophore, presumably aerobactin, compared with 6% of hydroxamate-producing isolates from nonanimal sources. In addition to *E. coli*, aerobactin is also produced by other human pathogens such as *Shigella flexneri*

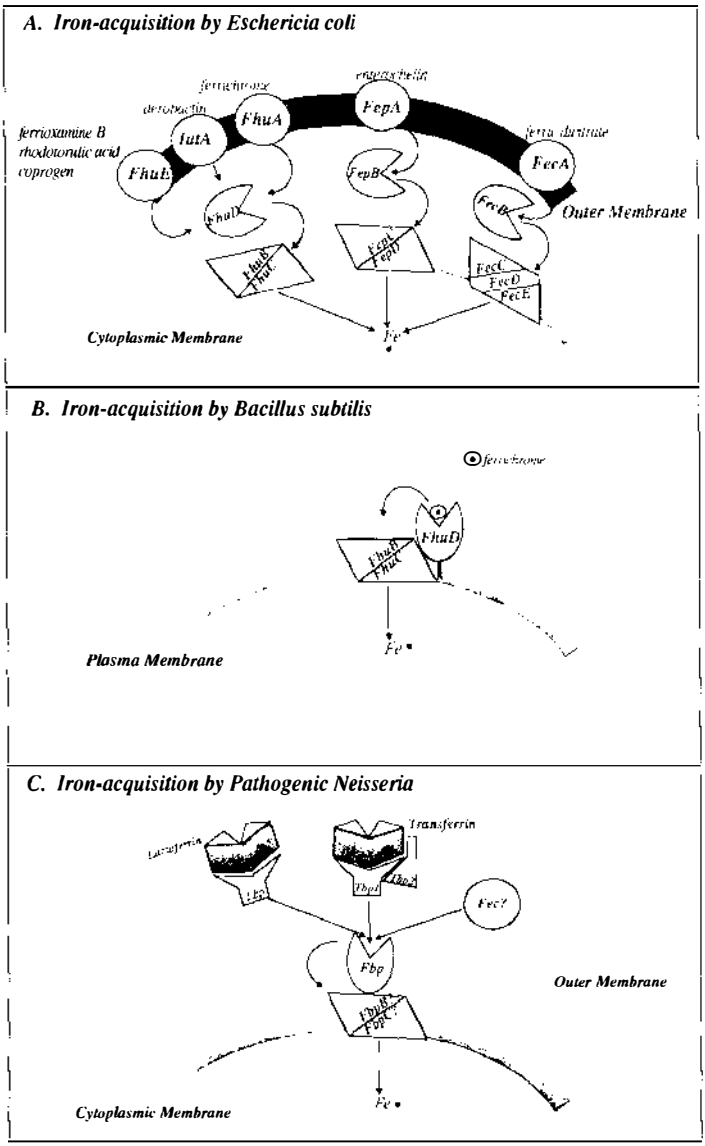

Figure 2 **Comparison of the gene products that facilitate transport of iron for *E. coli, B. subtillis,* and pathogenic *Neisseria spp.* Note that the general classes of proteins for gram-negative pathogens involve an outer-membrane receptor, a periplasmic binding component, a cytoplasmic permease, and a nucleotide-binding protein. In the case of the gram-positive organism, *B. subtillus,* which has no outer membrane, a lipoprotein on the external surface of the cell sharing homology with the periplasmic binding protein initiates iron-siderophore transport. The proteins facilitating iron acquisition are described in the text.**

(100) and *Enterobacter aerogenes* (35). The correlation between aerobactin production and bacterial infection suggests that this siderophore is important for the survival of these pathogenic microorganisms.

Studies of iron acquisition by *E. coli* have helped define the components required for the uptake and utilization of iron from ferri-siderophore complexes. Using high-resolution genetic analysis, these studies demonstrated that iron acquisition is accomplished by a generalized active transport process. As summarized for *E. coli* in Panel A of Figure 2, this process involves binding of the complex by a specific outer-membrane receptor, energy-dependent transport across the outer membrane, binding by a periplasmic binding protein, and transport across the cytoplasmic membrane by a specific permease using energy derived from a nucleotide-binding protein. For example, ferric enterochelin is bound by FepA, an integral outer-membrane protein (112). As summarized by Nikaido (78), FepA binds these complexes by surface exposed loops and transport occurs through a gated pore. The energy for transport is coupled through a TonB- and ExbB-dependent process (45, 50). Subsequent to transport of the ferric enterochelin complexes across the outer membrane, they are retained by a periplasmic binding protein, FepB (42, 95). Although direct interaction between FepB and ferrienterochelin has not been demonstrated, the specificity of this protein is generally considered to be similar to that of the well-characterized enteric periplasmic nutrient-binding proteins (7). The *fepC* gene product is peripherally associated with the cytoplasmic membrane and is thought to function as the nucleotide-binding protein that couples energy to a cytoplasmic membrane–associated permease encoded for by *fepD* (27, 95).

Depending on the isolate, *E. coli* may or may not synthesize aerobactin. However, all isolates typically utilize a number of fungal hydroxamate siderophores such as ferrichrome, coprogen, and rhodotorulic acid (35). Transport of these compounds is initiated by three distinct outer membrane receptors: FhuA for ferrichrome, IutA for ferri-aerobactin, and FhuE for coprogen and rhodotorulic acid. Upon binding and TonB-dependent translocation of each of these ferri-hydroxamates across the outer membrane, three gene products—FhuC, FhuD, and FhuB—are required for transport across the cytoplasmic membrane. Genetic and biochemical evidence has demonstrated that FhuD is the periplasmic binding component of this transport system. FhuD apparently exhibits much less specificity for the ferri-hydroxamate compounds than for the hydroxamate-specific outer membrane receptors. Likewise, FhuC and FhuB represent the nucleotide-binding protein and permease components, respectively, of hydroxamate transport across the cytoplasmic membrane.

A third high-affinity mechanism of iron acquisition by pathogenic *E. coli* involves uptake of ferric dicitrate complexes. At neutral pH, two molecules of

citrate chelate iron with an affinity of 1×10^{17}. An outer membrane receptor designated FecA specifically recognizes these complexes (47). As described above, transport across the outer membrane is TonB dependent. The periplasmic binding component of this system is thought to be FecB, since this protein is associated with the periplasmic space. Staudenmaier et al proposed that the nucleotide-binding component is the gene product of *fecE* and that *fecC* and *fecD* encode for cytoplasmic permease (98).

These studies show that *E. coli* requires the following general classes of proteins for iron acquisition: (*a*) a specific outer membrane receptor required for binding the iron complexes; (*b*) a component, e.g. TonB, that supplies the energy for transport of the iron complex across the outer membrane; (*c*) a periplasmic binding protein that binds the iron complex; (*d*) a cytoplasmic membrane–associated permease; and (*e*) a nucleotide-binding protein that supplies the energy for transport of the iron complex across the cytoplasmic membrane. These classes of proteins also applies to iron assimilation by other gram-negative bacteria. Gram-positive bacteria have only a single membrane and therefore require only the proteins to bind and transport iron complexes across this membrane. A recent report by Schneider & Hantke (89a) on the gram-positive organism *Bacillus subtillus* summarizes the utilization of hydroxamate siderophores. A protein (referred to as FhuD and sharing homology with the *E. coli* FhuD) is anchored to the cell surface via a covalently attached lipid and may be the lipated equivalent of a periplasmic binding protein (Figure 2, Panel B).

SIDEROPHILIN-MEDIATED IRON ACQUISITION The acquisition of iron by pathogenic microorganisms using siderophores has been well accepted since the early 1980s. However, another mechanism evolved based on studies of iron acquisition by the mucosal pathogens, *Neisseria meningitidis* and *Neisseria gonorrhoeae.* Norrod & Williams (79) and Archibald & DeVoe (4) failed to detect siderophore activity in culture supernatants of *N. gonorrhoeae* and *N. meningitidis*, respectively. However, these results were not immediately accepted because it was necessary to disprove the existence of a siderophore. This issue was resolved by Archibald & DeVoe (9), who sequestered ferritransferrin in a dialysis bag and immersed it in iron-limiting media followed by inoculation of the media outside the dialysis bag with either *E. coli* or *N. meningitidis.* They found that *E. coli* grew, whereas *N. meningitidis* did not. This observation led to the conclusion that transferrin binding was required for *N. meningitidis* but not for *E. coli,* because the latter synthesized siderophores capable of traversing the dialysis bag. Transferrin-binding activity was iron regulated because transferrin binding increased in response to growth under conditions of iron deprivation (96).

McKenna et al reported similar transferrin-binding activity for *N. gon-*

orrhoeae (64). All strains of *N. gonorrhoeae* and *N. meningitidis* bind and utilize iron from human transferrin (65, 66). A pleiotropic mutant of *N. meningitidis* obtained by a combination of chemical mutagenesis and streptonigrin enrichment (38, 39) bound transferrin but could not utilize the iron from this source. A subsequent report (103) estimated that iron-starved meningococci had a receptor density of 2900 molecules per cell and a K_{diss} of 0.7 μM for human transferrin. It was also determined that ^{125}I-labeled *apo*-transferrin bound to the meningococcal transferrin receptor with the same affinity as ^{125}I-labeled iron-saturated transferrin in a solution-phase assay. These results contradict those of Schryvrers & Morris (94), who used a solid-phase assay with whole cells and observed that a 10-fold excess of *apo*-transferrin competed with iron-saturated transferrin for binding. This discrepancy is important because it leads to the prediction that rapid dissociation of the *apo*-transferrin molecule from the receptor facilitates the efficient acquisition of transferrin-bound iron, as is the case for the eukaryotic transferrin receptor (32). Conversely, the meningococcal transferrin receptors are saturable at 1 μM transferrin, a concentration 30-fold less than that of transferrin in serum and 10-fold less than the molar concentration of iron bound to human transferrin. Because serum iron bound to human transferrin is in excess of the saturable meningococcal transferrin receptors, turnover may not be an issue. Transferrin bound to the meningococcal or gonococcal cell surface may also act as a bridging ligand to the eukaryotic transferrin receptor and facilitate attachment and uptake. High concentrations of transferrin receptors are found on endothelial cells in the brain, which are involved in the transcytosis of transferrin to the cerebrospinal fluid (61). This binding may provide a mechanism of entry for bacterial pathogens such as *N. meningitidis.*

Mammalian cells can remove iron from transferrin via receptor-mediated endocytosis (117). Binding of transferrin by its receptor is followed by internalization of the receptor-protein complex, removal of iron from transferrin within cellular endosomes, and extracellular release of *apo*-transferrin by the cell. However, endocytosis does not occur in bacteria. Transport of iron from the sidero- philins is therefore distinct from the mammalian process and is currently not well understood. We do know that the N-linked oligosaccharides of human transferrin are not required for binding to bacterial transferrin receptors (81), which suggests that the protein moieties of this molecule mediate binding. Alcantara et al (4) digested iron-saturated human transferrin with either trypsin or chymotrypsin to produce C-lobe and N-lobe protein fragments and purified them by a combination of gel filtration and concanavalin A chromatography. These investigators obtained evidence that the C-lobe of human transferrin is involved in binding to the meningococcal transferrin receptor.

All transferrins, even those from phylogenetically distant species, share considerable sequence homology (32). Mammalian transferrin receptors can

bind transferrin from several species, as inferred by the ability of fetal calf serum to support growth of human cells, for example, in tissue culture. This binding is probably mediated through phylogentically conserved domains (4, 14), in contrast to bacterial transferrin receptors, which have a strict species specificity (112). For example, *N. meningitidis* and *N. gonorrhoeae* neither bind nor utilize iron bound to bovine or porcine transferrin (89b, 94), whereas human transferrin is both bound and utilized as a source of iron. Recently, it was reported (4) that *N. meningitidis* bound transferrin from primate representatives of the hominid lineage (chimpanzee, gorilla, orangutan) but not transferrins from old- and new-world monkeys. These results reflect a phylogenetic relationship to humans and may explain why only humans, chimpanzees, orangutans, and presumably gorillas can be naturally infected with *N. gonorrhoeae* (10).

The molecular basis for transferrin receptor–binding activity has been well studied for *N. gonorrhoeae* and *N. meningitidis* (112). For example, Schryvers and colleagues (94) showed that iron-starved meningococci or gonococci immobilized on a nitrocellulose membrane-bound horseradish peroxidase (HRP) conjugated human transferrin. Upon incubation of cell membranes from iron-starved organisms with biotinylated human transferrin followed by detergent solubilization, these investigators could affinity purify specific proteins (operationally defined as transferrin-binding proteins, Tbps) with molecular masses of ~ 100 kDa (designated Tbp1) and ~ 70 kDa (designated Tbp2) (89b). Subsequent studies indicated that the molecular mass of Tbp2 was strain dependent and varied from 64 to 85 kDa (86, 112). The genes encoding the meningococcal Tbp1 and Tbp2 have been cloned and sequenced and are now referred to as *tbp*A and *tbp*B, respectively (55). Gonococcal homologues of Tbp1 and Tbp2 have also been demonstrated (89b) and were recently cloned (31). By sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the estimated molecular masses of the gonococcal Tbp1 and Tbp2 were 100 kDa and 85 kDa, respectively (29). Sequence analysis of Tbp1 revealed homology with the TonB-dependent outer membrane receptors fepA, fecA, iutA, fhuA, and fhuE (29). This finding implies that a TonB analog must exist in pathogenic *Neisseria* species.

Irwin et al (55) constructed isogenic mutants in the *tbpA* and *tbpB* genes. Isogenic mutants deficient in Tbp1 or Tbp2 exhibited reduced binding of transferrin by whole cells and total membrane preparations and could not utilize transferrin for growth. These results indicate that both proteins are involved in the utilization of transferrin by *N. meningitidis.* Ala'Aldeen et al (2) demonstrated by electron microscopic examination of meningococci incubated with gold-labeled human transferrin that Tbps are surface exposed. Palmer et al (82) cloned the entire meningococcal Tbp1 gene in *E. coli.* The Tbp1 was surface exposed on *E. coli* and bound human transferrin, as dem-

onstrated by electron microscopy with gold-labeled human transferrin. However, evidence from Rokbi et al (86) indicates that although Tbp2 is accessible on the surface of the bacteria, Tbp1 may not be. This surface accessibility agrees with the antigenic variability of Tbp2, since exposure to the immune system of the host would lead to selection of antigenic variants.

All strains of *N. meningitidis* can utilize human lactoferrin-bound iron for growth (65). Studies of the utilization of lactoferrin-bound iron by gonococci have produced conflicting evidence. Mickelsen et al (65) observed that only 29 of 57 strains of *N. gonorrhoeae* could utilize lactoferrin-bound iron for growth; 24 of the 28 strains incapable of utilizing lactoferrin-bound iron belonged to the $Arg^-Hyx^-Ura^-$ (AHU) auxotype. These strains have been associated with asymptomatic uncomplicated gonorrhea (31) as well as with disseminated infections (58). Lee & Bryan (60) used a solid-phase binding assay to quantitate lactoferrin binding by various strains of *N. gonorrhoeae.* All strains, regardless of auxotype, bound human lactoferrin and could utilize lactoferrin-bound iron for growth; however, AHU strains were not examined. Instead, these investigators used $Orn^-Hyx^-Ura^-$ strains as a surrogate marker for dissemination and found that they expressed low levels of lactoferrin receptor activity. Lactoferrin concentration in human vaginal mucus varies during the menstrual cycle (28). Thus, low levels of lactoferrin coupled with decreased receptor density may limit the growth of some strains of *N. gonorrhoeae.*

Lactoferrin binding and transferrin binding by *N. meningitidis* and *N. gonorrhoeae* exhibit similarities as well as differences. Lactoferrin binding is specific for human lactoferrin (92) and is not dependent on the level of iron saturation (93). The N-linked oligosaccharides of human lactoferrin are also not required for binding (3). Blanton et al (20) obtained genetic evidence that *N. gonorrhoeae* produces specific receptors for lactoferrin and transferrin. Moreover, acquisition of lactoferrin- and transferrin-bound iron appeared to be linked through a nonreceptor gene product essential for obtaining iron from both of these sources. These results corroborated those of competition-binding assays in which human lactoferrin—not human transferrin—blocked binding of HRP-conjugated human lactoferrin (93). The level of iron in the medium regulated the expression of lactoferrin-binding activity. The lactoferrin-binding protein was identified as an outer-membrane protein with a molecular mass of 105 kDa in both *N. meningitidis* and *N. gonorrhoeae* (60, 89b, 90, 93, 94).

Subsequent to binding of iron transferrin to its gonococcal receptor, iron is transported onto the 37 kDa major iron-regulated protein, ferric iron–binding protein (Fbp) (26). All pathogenic *Neisseria spp.* produce Fbp (67, 69, 70). Fbp has been purified to homogeneity (68), cloned and sequenced (16, 17), and overexpressed in *E. coli* (15). It reversibly binds one molecule of ferric iron per molecule of protein (68). Under acidic conditions (pH 4.5) and in the

presence of sufficient quantities of an appropriate iron chelator (e.g. citrate), iron can be removed from Fbp. Upon neutralization, iron is rapidly rebound by Fbp, with an affinity for Fe^{3+} of ~ 1×10^{-19} lM^{-1}, which is on the order of the affinity of the siderophilins for ferric iron. Fbp was recently localized to the periplasm of pathogenic *Neisseria* (15) and found to transiently accept iron from transferrin (26). These properties implicate Fbp in Neisserial iron acquisition as a classic periplasmic binding protein. Panel C of Figure 1 shows the current model for iron acquisition by pathogenic *Neisseria.* However, the physical existence of a cytoplasmic permease and nucleotide-binding protein for pathogenic *Neisseria* has not been determined. Moreover, no one has identified a naturally occurring isolate or obtained a gonococcal or meningococcal mutant deficient in the production of Fbp. This observation suggests that Fbp may be essential for the survival of these bacteria.

Siderophilins bind to the surfaces of other microorganisms that are pathogenic for humans. Like pathogenic *Neisseria, Haemophilus influenzae* binds and utilizes iron from human transferrin by a siderophore-independent mechanism (72, 90). Binding is not limited to human transferrin; bovine transferrin was bound, although less efficiently (72). *H. influenzae* also binds lactoferrin but cannot utilize this siderophilin as an iron source (90). Schryvers and colleagues characterized the transferrin and lactoferrin receptors in *H. influenzae* (90, 91) and found them to be analogous to those reported in *Neisseria spp.* An iron-regulated periplasmic protein with substantial amino acid sequence homology with the Neisserial Fbp has also been described in *H. influenzae* (51). *Bordetella pertussis* binds human transferrin and lactoferrin and can utilize this siderophilin-bound iron for growth (84, 85). However, *B. pertussis* also produces a hydroxamate siderophore. Direct contact of transferrin with a receptor does not appear to be essential for iron acquisition by *B. pertussis,* but it is far more effective than siderophore activity alone.

In some instances, binding is specific for human siderophilins but is not associated with the utilization of siderophilin-bound iron. For example, human lactoferrin binds to the outer membrane porin protein of *Aeromonas hydrophila* (57) and *Shigella flexneri* (102). In addition, human lactoferrin binds to the cell envelope and inhibits the growth of the oral microorganisms *Prevotella intermedia, Prevotella melaningencia,* and *Porphyromonas gingivalis* (56). Relatively few organisms specifically bind and utilize the iron from human siderophilins. Binding of human lactoferrin has also been reported for *Mycoplasma pneumoniae* (104), the spirochete *Treponema pallidum* (5), and the protozoan *Trichomonas vaginalis* (83).

H. pylori is a microaerophilic, spiral, gram-negative bacterium found exclusively in the human gastric mucosa (114), where it is exposed directly to the ~ 10 mg of iron ingested by the human host on a daily basis (109). *H. pylori* may not exist within an iron-limited but rather in an iron-sufficient environ-

ment. These organisms do not secrete siderophores and cannot utilize enterochelin, human transferrin, bovine lactoferrin, or *ovo*-transferrin for growth (54). However, human lactoferrin can fully support the growth of *H. pylori* in iron-limited media. Lactoferrin is found in large quantities in stomach resections and may play an important role in the pathogenesis of *H. pylori* infection (54). This pathogen reportedly expresses a 19.6-kDa iron-binding protein in large quantities (37). It has been cloned and sequenced and shares impressive homology with human ferritin (44). In this capacity, the iron-binding protein may detoxify the iron absorbed by *H. pylori* in vivo.

HEME IRON ACQUISITION *H. influenzae* requires exogenously supplied heme and nicotinamide adenine dinucleotide (NAD) (classically referred to as the X and V factors, respectively) for growth in vitro. The basis for this heme requirement is the inability to convert δ-aminolevulinic acid to protoporphyrin (49). Perhaps in response, these organisms have developed an efficient mechanism for binding and transport of heme into the cell. Growth in vitro of these organisms can be accomplished using heme as a sole source of iron (80). Coulton & Pang (30) demonstrated that both iron and the porphyrin ring are taken up at the same rate. Potential heme-binding proteins of 43 kDa (30) and 51 kDa (48) have been reported. Pathogenic *Neisseria spp.* and *E. coli* can also utilize heme as a sole source of iron (66, 80, 116), and candidate receptor proteins that initiate this process by binding heme have been reported (59).

The majority of free extracellular heme is bound to albumin or hemopexin; heme associated with extracellular hemoglobin is bound to haptoglobin. *N. gonorrhoeae* cannot utilize heme bound to hemopexin or to albumin (40), and *E. coli* cannot utilize heme iron from hemoglobin/haptoglobin complexes (41). In contrast, *H. influenzae* can utilize heme from all of these sources (99). The in vivo source of heme for *H. influenzae* is not known, but the ability to scavenge heme in vivo seems to confer a considerable advantage to these organisms. Recently, Hanson et al (49) identified and characterized a genetic locus in *H. influenzae* type b that was necessary for the binding and utilization of heme bound to human hemopexin. The protein encoded by this gene has a molecular mass of ~ 100 kDa, was localized to the *H. influenzae* cell surface, and was required for utilization of heme iron from hemopexin. Another study by Wong et al (113) reported hemopexin-binding proteins with molecular masses of 57, 38, and 29 kDa based on affinity chromatography of detergent-solubilized cell envelopes of *H. influenzae* grown aerobically under conditions of iron deprivation. In contrast to the transferrin receptors expressed by this organism and by pathogenic *Neisseria,* the hemopexin binding did not distinguish human hemopexin from rabbit hemopexin, even though *H. influenzae* is exclusively a human pathogen (113).

Porphyromonas (Bacteroides) gingivalis requires heme for growth but can be efficiently cultivated in vitro in the presence of haptoglobin, hemopexin, or human serum albumin. Transferrin and lactoferrin can also serve as iron sources for this organism. Furthermore, no siderophore has been identified for this organism. When grown under hemin-starved, iron-starved conditions, this organism expressed a major 26-kDa protein that was surface exposed (21). This protein continued to be expressed when *P. gingivalis* was grown under hemin-starved but iron-sufficient conditions. Only when organisms were propagated in the presence of hemin proficiency could the synthesis of this protein be repressed. Bramanto & Holt speculate that this protein may play a role in the transport of both growth-essential heme and growth-essential iron (21).

Shigella spp. are enteroinvasive pathogens that penetrate and multiply within intestinal epithelial cells. The intracellular environment may be a particularly important source of heme. Studies have shown a correlation between binding of Congo red (a hemin analog), binding of hemin, and virulence of *Shigella spp.* (36). Binding of Congo red and of hemin correlates with the presence of a plasmid-encoded cell-surface protein of 101 kDa (100). However, the presence of this protein did not correlate with heme iron assimilation; rather, it was speculated that *Shigellae* coated with heme may utilize host heme receptors for efficient endocytosis and invasion (80). *Yersinia pestis* expresses neither a classical siderophore nor a siderophilin receptor but can obtain iron from ferritin, hemin, hemin complexed to hemopexin, hemoglobin, haptoglobin-hemoglobin, and myoglobin (97). The mechanism of iron acquisition from these sources is not understood but may involve iron-regulated proteins of high molecular weight (97). The ability to utilize these iron sources may in part explain the predilection of hyperferremic patients for contracting yersiniosis (46).

Expression of Iron-Binding Proteins in vivo

Our understanding of the iron-binding proteins involved in iron acquisition by pathogenic microorganisms is largely limited to the examination of proteins expressed under well-defined in vitro systems. Little is known about the levels of expression of these proteins during the course of infection. In this regard, several studies have measured antibody response to iron-regulated proteins as an indicator for expression of these proteins in vivo. For example, Fohn et al (43) analyzed the immune response to the gonococcal Fbp from patients with uncomplicated and disseminated gonococcal infection and reported that one in nine patients suffering from uncomplicated infection produced antibody to Fbp. In contrast, all patients suffering from disseminated infection produced antibodies to Fbp (43). These results suggested that this iron-regulated protein was both expressed and immunogenic in vivo, particularly during invasive

disease. Black et al (19) studied the immune response to the iron-repressible outer-membrane proteins of *N. meningitidis* and demonstrated that antisera from patients convalescing from meningococcal infection recognized the iron-regulated outer-membrane proteins of high molecular weight. Their findings also suggested that these proteins were expressed in vivo. More recent studies analyzed the transferrin-binding proteins of *N. meningitidis* as potential components of a noncapsular polysaccharide-based vaccine for the prevention of meningococcal meningitidis (13), thereby underscoring the importance of these proteins in the survival of pathogenic bacteria.

CONCLUSIONS

The survival of bacterial pathogens within the human host depends in part on the efficient scavenging of growth-essential iron. Pathogenic microorganisms employ a variety of different strategies for obtaining iron. For example, pathogens such as *E. coli* cause disease within the gastrointestinal tract or use this environment as a staging ground to gain access to and cause invasive disease in sterile compartments of the human host. These organisms are typically transmitted by a mechanism that requires growth outside of the human host. Therefore, the source of their iron may vary considerably, ranging from the human gut to the environment. Consequently, *E. coli* may have evolved the inefficient mechanism of siderophore-mediated iron acquisition that does not discriminate much among iron sources. In contrast, organisms that are dedicated human pathogens, such as the pathogenic *Neisseria spp.*, appear to be addicted to the iron resources of the human host and utilize a much more efficient yet host-limiting strategy: They essentially replace the siderophore with the host siderophilin as the iron source. Other pathogens may use entirely different methods to acquire growth-essential iron. For example, they may obtain this element in the form of heme or another intracellular target. The source of host iron for which these pathogens are expected to compete within their given niche may well determine which approach they take.

When examining the potential role of iron-binding proteins in the survival of pathogenic microorganisms, one must consider the source of the iron, its use by the bacteria, and how the pathogen obtains the iron. In this regard, although much effort has been devoted to determining how the microorganism obtains iron from the host environment, the specific iron source of the host is equally important. The characteristics distinguishing a pathogen from a related commensal or environmental microorganism are generally but not always far more complex than a single gene or attribute. In this context, highly efficient iron-acquisition systems (i.e. iron-binding proteins) are essential to the survival of pathogenic bacteria because of the physiologic relevance of iron in the multiplication of bacteria.

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