Iron Acquisition Strategies of Bacterial Pathogens

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ABSTRACT Iron is an essential micronutrient for both microbes and humans alike. For well over half a century we have known that this element, in particular, plays a pivotal role in health and disease and, most especially, in shaping host-pathogen interactions. Intracellular iron concentrations serve as a critical signal in regulating the expression not only of high-affinity iron acquisition systems in bacteria, but also of toxins and other noted virulence factors produced by some major human pathogens. While we now are aware of many strategies that the host has devised to sequester iron from invading microbes, there are as many if not more sophisticated mechanisms by which successful pathogens overcome nutritional immunity imposed by the host. This review discusses some of the essential components of iron sequestration and scavenging mechanisms of the host, as well as representative Gram-negative and Gram-positive pathogens, and highlights recent advances in the field. Last, we address how the iron acquisition strategies of pathogenic bacteria may be exploited for the development of novel prophylactics or antimicrobials.

BIOLOGICAL IMPORTANCE OF IRON
Iron is essential to nearly all life forms on Earth, required for the proper function of enzymes involved in, for example, respiration, photosynthesis, the tricarboxylic acid cycle, nitrogen fixation, electron transport, and amino acid synthesis. The utility of iron in biological processes hinges on its chemical properties as a transition metal, engaging in single electron transfers to interconvert between the ferrous (Fe²⁺) and ferric (Fe³⁺) states. While this clearly makes iron advantageous, the same property provides the explanation for why excess, or “free,” iron is inherently toxic. Ferrous iron–catalyzed Fenton chemistry results in the generation of the highly toxic hydroxyl radical (OH•) that can compromise cellular integrity through damage to lipids, proteins, and nucleic acids.

Aside from Borrelia burgdorferi and Treponema pallidum, iron is essential to all microbial pathogens, yet perhaps the most difficult issue facing pathogens is accessing enough iron to support growth. The concentration of iron under physiological conditions (10⁻⁸ to 10⁻⁹ M) is orders of magnitude below the ∼10⁻⁶ M required for bacterial growth, owing to the formation of insoluble ferric oxyhydroxide precipitates, and host sequestration mechanisms further decrease the available concentration to the range of ∼10⁻¹⁸ M. As such, iron plays a fundamental role in host-pathogen interactions, and coevolution has shaped both bacterial and host iron acquisition/sequestration mechanisms.

IRON METABOLISM IN HEALTH AND DISEASE
Host Iron Homeostasis Perturbations to the balance of iron within the human body can impact both overall health and susceptibility to infectious disease. Sophisticated mechanisms thus exist to control the daily intake of iron that is required for metabolic processes and the synthesis of
new erythrocytes. The majority of iron within the body is found intracellularly, in association with heme, a planar tetrapyrrole ring that coordinates a central ferrous iron ion, and is bound within proteins such as hemoglobin or myoglobin. Hemoglobin is typically contained within circulating erythrocytes, but during routine destruction of senescent cells, it is released into the blood. Although the levels of extracellular hemoglobin are usually low, rapid erythrocyte lysis triggered by pathological conditions can increase the concentration of free heme and hemoglobin to harmful levels. Free hemoglobin is thus scavenged by haptoglobin, whereas hemopexin is involved in sequestration of free heme. Through receptor-mediated endocytosis, hemoglobin-haptoglobin and heme-hemopexin complexes are taken up by macrophages or hepatocytes, and the bound iron can either be stored or returned to the iron cycle.

Dietary iron is taken up by enterocytes as either heme-iron or as ferrous iron through apical surface localized heme carrier protein (HCP)-1 or diveral metal transporter (DMT)-1, respectively. Ferric iron is first reduced to ferrous iron prior to uptake, and heme is degraded by heme oxygenase to release ferrous iron. Ferrous iron faces three potential fates inside the enterocyte: (i) utilization in cellular processes, (ii) sequestration in the multimeric, iron-storage protein, ferritin, or (iii) export from the cell into the circulation via ferroportin. Ferroportin is the only known iron exporter in humans, and it functions to mobilize iron primarily from enterocytes and macrophages for transport to sites of demand within the body. Ferrous iron efflux by ferroportin is oxidized by hephaestin on the surface of enterocytes, and ceruloplasmin on nonintestinal cells and within the plasma, thus permitting iron-loading of transferrin, which has poor affinity for Fe\(^{3+}\).

**Iron-Withholding as a Facet of Innate Immunity**

Transferrin is an abundant serum glycoprotein capable of reversibly coordinating two molecules of Fe\(^{3+}\) with very high affinity (K\(_d\) = 10\(^{-23}\) M\(^{-1}\) at neutral pH). Transferrin is invaluable to iron homeostasis in humans, because it delivers iron to various cells but also scavenges free iron in the bloodstream, essentially sequestering it from invading pathogens. The transferrin-like glycoprotein lactoferrin is commonly found extracellularly in secretions and intracellularly in the secondary granules of neutrophils. Lactoferrin functions to sequester iron at mucosal surfaces and is released from neutrophils at infectious foci, thus participating in the host defense against invading microbes.

Many dynamic processes exist within the host to restrict the iron available to pathogens. The withholding of iron, and other essential metals, is referred to as “nutritional immunity” and functions as a key component of innate immunity. Much of this response is orchestrated by the human hormone hepcidin, which is secreted by the liver and directly regulates the internalization and degradation of ferroportin. Hepcidin is released primarily in response to excess levels of extracellular iron, either to combat transferrin saturation or as part of the inflammatory response. With the degradation of ferroportin, iron is stored intracellularly, dietary iron uptake is halted, and iron release from macrophages is stopped. Overall, this process may result in a decrease in serum levels by as much as 30%, and when triggered by invading microbes, is referred to as the “hypoferremia of infection.”

To combat infection by intracellular pathogens, the natural resistance macrophage protein 1 functions to export iron and other essential metals from the phagosomal compartment. Natural resistance macrophage protein 1 is also involved in the release of neutrophil gelatinase-associated lipocalin (NGAL; also known as siderocalin, lipocalin 2, and 24p3), an acute-phase protein secreted primarily by neutrophils that scavenges catechol-type siderophores produced by bacteria. NGAL binds enterobactin, a siderophore widely synthesized by the *Enterobacteriaceae*, and NGAL-deficient (NGAL\(^{-/-}\)) mice are hypersusceptible to Gram-negative bacterial infections (1). Despite lacking an intrinsic ability to bind iron, NGAL appears to function primarily in the maintenance of extracellular and intracellular iron concentrations within the host. The transport of iron by NGAL is purportedly aided by the mammalian siderophore, 2,5-dihydroxybenzoic acid (2,5-DHBA), which is structurally similar to the 2,3-DHBA iron-binding moiety of enterobactin (2, 3). 2,5-DHBA likely scavenges free iron and, when bound to NGAL, may be effectively shuttled across cellular membranes (2). Notably, 2,5-DHBA is capable of promoting growth of *Escherichia coli* in a mammalian model, so it is not surprising that synthesis of 2,5-DHBA is downregulated during infection, freeing NGAL to bind to bacterial siderophores.

**Hereditary Diseases Compromising Iron-Withholding Within the Host**

Although iron homeostasis is stringently controlled, many hereditary disease states, as well as excess expo-
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sure to the element, can result in an imbalance in iron levels, impacting both the overall health of the host and susceptibility to infectious disease. The most common genetic disease influencing host iron metabolism is hereditary hemochromatosis, which is largely caused by defects to the gene encoding human hemochromatosis protein, HFE (for high Fe). Although the exact molecular mechanisms governing hereditary hemochromatosis are still unclear, recent insights suggest that HFE is involved in iron-sensing and the regulation of hepcidin production (4). HFE is thought to participate in the formation of an iron-sensing complex, which in the presence of high levels of holo-transferrin activates a signal cascade promoting transcription of the gene encoding hepcidin (HAMP) (5). Deactivation of this regulatory complex and/or cascade results in inadequate production of hepcidin, leading to excessive intestinal iron absorption and recycling (6). The net result of the aforementioned dysregulation is abnormally high levels of free iron within the body, broadly referred to as iron overload.

Other less common hereditary iron overload diseases are principally associated with genetic defects in the genes encoding ceruloplasmin (CP) and transferrin (TF), which result in aceruloplasminemia and transferrinemia, respectively (7). The absence of ceruloplasmin impairs the ability of ferroportin to export iron in cells lacking an alternate ferroxidase (e.g., hephaestin), as is the case with astrocytes of the central nervous system, which leads to iron accumulation in the brain and progressive neurodegeneration (8). Reduced or absent serum transferrin similarly impairs the trafficking of iron throughout the body, as well as the production of hepcidin (as discussed above), leading to accretion primarily within the liver and heart. Further, inadequate iron delivery to hematopoietic cells in the bone marrow by transferrin results in decreased hemoglobin availability, leading to anemia (9). The symptoms of hereditary hemochromatosis and aceruloplasminemia are most effectively delayed or reduced through phlebotomy and/or chelation therapy (5, 10), while transferrinemia/acytansferrinemia is managed primarily through transfusion of whole blood or purified apo-transferrin (11). Not only do diseases impacting host iron homeostasis enhance the risk of severe and invasive infections, but often so do their clinical interventions. Indeed, while thalassemia and sickle cell disease (SCD) can result in iron overload, it is often the treatment for these conditions that leads to hyperferremia. Both thalassemia and SCD are characterized by the abnormal formation of adult hemoglobin, a heterotetramer comprised of two α-globin subunits and two β-globin subunits. In thalassemia, genetic defects can impact the synthesis of either of the globin subunits (α-thalassemia and β-thalassemia), resulting in ineffective erythropoiesis and accelerated hemolysis of existing erythrocytes (12). SCD is due specifically to a single amino acid change in the β-globin subunit, which results in the polymerization of hemoglobin tetramers into rigid fibers and gives rise to the sickle-shaped erythrocytes from which the disorder derives its name (13, 14). Sickle cells are inherently fragile, and their instability contributes to the development of hemolytic anemia. As with thalassemia, the symptoms of SCD may be treated with frequent blood transfusions, which enhances not only the risk of iron overload, but also the frequency and severity of bacterial and fungal infections.

To reduce iron content in patients suffering iron overload, treatment can include phlebotomy or chelation therapy. Deferoxamine mesylate, the mesylate salt derivative of the microbial siderophore desferrioxamine B, is frequently used for chelation therapy. Deferoxamine mesylate, however, can promote more severe infection by some bacteria, including Yersinia spp., Klebsiella spp., and Staphylococcus aureus (15–17), which are able to use the siderophore as a source of iron.

OVERVIEW OF BACTERIAL IRON ACQUISITION STRATEGIES

Even within the healthy host, bacterial invaders that scavenge enough iron have the potential to cause infection. Iron has ultimately driven an evolutionary arms race between microbes in pursuit of this essential element and hosts striving to withhold it from them. In effect, for every mechanism employed by the host to sequester iron, bacterial pathogens have evolved mechanisms to circumvent this withholding strategy (see Fig. 1). Accordingly, a diverse array of iron acquisition strategies exist among bacterial pathogens, some of which are highly conserved and broadly employed, while others are highly specific and intricately linked to the pathophysiology of a given bacterium. Broadly, and as summarized in Fig. 1, the mechanisms for iron uptake by pathogenic bacteria include (i) the extraction and capture of heme-iron from host hemoproteins through the use of secreted proteins or cell surface-associated receptors, (ii) the acquisition of transferrin and lactoferrin-bound iron through specific surface-associated binding proteins or the secretion of siderophores, and (iii) the uptake of free inorganic iron facilitated by ferric iron reductases and associated ferrous iron permeases. Later in the chapter, the mechanisms by which pathogens acquire iron within the host.
are discussed, with a focus on how these mechanisms are known to impact the virulence of bacterial pathogens.

**Coordinated Regulation of Iron Acquisition and Virulence Gene Expression**

Iron, or lack thereof, is a fundamental sensory cue in bacterial pathogens, and it triggers the coordinated regulation of genes involved in both iron acquisition and virulence. The response to iron deprivation in prokaryotes is largely controlled by two separate families of highly conserved, iron-responsive regulators that repress transcription of iron acquisition genes when the concentration of intracellular iron is high and alleviate this repression when iron is limiting. In Gram-negative bacteria, and the low–guanine and cytosine (G+C)-content Gram-positive Firmicutes, such as *Staphylococcus* spp., *Bacillus* spp., and *Listeria monocytogenes*, iron homeostasis is controlled by members of the ferric uptake regulator (Fur) superfamily (18). The high-G+C-content Gram-positive *Actinobacteria*, *Corynebacterium diphtheriae*, and *Mycobacterium* spp., utilize an alternative iron-responsive regulator, the diphtheria toxin repressor (DtxR; IdeR in the *Mycobacteriaceae*) to mediate both iron uptake and virulence gene expression (19). Notably, while little primary sequence similarity exists between members of these two families, Fur and DtxR-like proteins possess similar domain architecture and function in a comparable manner. In brief, both Fur and DtxR are homodimeric metalloregulators that bear an N-terminal helix-turn-helix DNA-binding domain and a C-terminal metal corepressor binding site that also functions in dimerization of the protein (18, 20). Coordination of Fe²⁺ within each protein subunit induces a conformational change rendering the iron-loaded repressor proficient for DNA binding, which occurs at a consensus sequence located within the promoter/operator region of the targeted gene (18, 21–23). Association of Fur-Fe²⁺ or DtxR-Fe²⁺ with the operator of iron-responsive genes effectively bars RNA polymerase from binding the promoter, thereby inhibiting transcription under iron-replete conditions. When intracellular iron concentrations are low, the metal is no longer readily available to interact with the repressor, the complex dissociates from DNA, and transcription proceeds.

The overwhelming majority of iron acquisition strategies employed by bacterial pathogens, and discussed herein, are wholly or at least partially regulated by members of the Fur or DtxR superfamilies. In addition to their role in iron acquisition, Fur and DtxR both play a key role in virulence and have been shown to directly or indirectly (e.g., through small Fur/DtxR-regulated RNAs) control the expression of factors contributing to pathogenicity, including the secretion of toxins, production of adhesins, formation of biofilms, and regulation of quorum sensing (24, 25). Indeed, DtxR was initially identified, as its name implies, as an iron-dependent negative regulator of diphtheria toxin production in *C. diphtheriae* (22, 26). Further, both Fur and DtxR have been demonstrated, through relevant *in vivo* models, to influence the colonization, survival, and/or proliferation of numerous bacterial pathogens within the host including, but not limited
accession (27). The role of these metalloregulatory proteins in vivo no doubt is complex and multifactorial, but it appears to reflect a common strategy employed by bacterial pathogens in order to respond appropriately upon sensing their transition into the iron-limited host.

**ACCESSING HEME IRON**

While bacterial pathogens express a plethora of iron acquisition systems, capable of exploiting a multitude of host iron sources, heme represents a particularly auspicious target because it comprises approximately 75% of the total mammalian iron pool. Indeed, heme is a preferred iron source for pathogens such as *S. aureus* (28) and an obligate requirement for heme auxotrophs including staphylococcal small colony variants (29), *Bartonella* spp., *Bacteroides* spp., *Porphyromonas gingivalis*, and *H. influenzae* (30). Because heme exists primarily in association with hemoglobin within circulating erythrocytes, invading pathogens have evolved sophisticated mechanisms to access heme from intracellular hemoproteins. The secretion of hemolysins is a tactic commonly employed by extracellular and facultative intracellular pathogens to enhance local heme availability. Indeed, the expression of such cytolytic factors is often induced under conditions of iron starvation and during bacteremia and has long been recognized as an important multifactorial determinant of pathogenicity in many bacteria (31).

Upon its release from damaged erythrocytes, hemoglobin, or spontaneously dissociated heme, may be captured by bacteria expressing specific secreted and/or cell surface–associated heme/hemoglobin-binding proteins. Additionally, some pathogens are capable of extracting heme from heme-iron complexes such as heme-hemopexin, hemoglobin-haptoglobin, and serum albumin, thus subverting the efforts of these host iron sequestration proteins in witholding iron from microbial invaders. In this section, archetypical heme acquisition systems will be discussed, as will be less-conserved methods employed by specific pathogens (see also Table 1).

**Extracellular Mechanisms of Heme Capture**

In a number of bacterial pathogens, the acquisition of extracellular heme as a cofactor or iron source is initiated by the synthesis and secretion of small, soluble, heme-binding proteins referred to as hemophores (32). Hemophores are capable of both capturing free heme and appropriating it from various hemoproteins. Hemophore-bound heme is subsequently transferred to specific heme-binding receptor proteins localized on the outer membrane in Gram-negative bacteria or the cell wall in Gram-positive bacteria, both of which help mediate uptake through the cell envelope. To date, two major types of hemophores have been characterized: the HasA-type hemophores of Gram-negative pathogens and the near iron transporter (NEAT)–domain containing hemophores of Gram-positive pathogens. Additionally, the heme auxotrophic bacteria *H. influenzae* and *P. gingivalis* each produce a distinct heme-binding protein, HxuA and HmuY (33, 34), respectively, to help fulfill their obligate metabolic requirements for heme. Each hemophore possesses unique mechanisms for export, acquisition of heme, and transfer of heme to the cell surface prior to uptake.

**HasA-type hemophores of Gram-negative pathogens**

HasA (for heme acquisition system) of *Serratia marcescens* was the first extracellular heme-binding protein to be isolated and characterized within the bacterial domain (35). HasA-homologous hemophores have since been identified in a number of pathogenic or opportunistically pathogenic bacteria including *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, *Yersinia enterocolitica*, and the soft rot-causing plant pathogens *Pectobacterium carotovorum* and *Pectobacterium atrosepticum* (32). HasA-type proteins lack significant sequence homology to other known proteins, and indeed to other types of hemophores, but display a high degree of structural conservation within the family. HasA of *S. marcescens*

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**TABLE 1** Examples of heme acquisition systems essential for virulence

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Heme system</th>
<th>Modela</th>
<th>Referenceb</th>
</tr>
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<tbody>
<tr>
<td>Bacillus anthracis</td>
<td>Isd</td>
<td>Mouse (i.t.)</td>
<td>275</td>
</tr>
<tr>
<td>Bordetella pertussis</td>
<td>Bhu</td>
<td>Mouse (i.n.)</td>
<td>276</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Chu</td>
<td>Mouse (i.t.)</td>
<td>277</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>Hbp</td>
<td>Mouse (i.p.)</td>
<td>278</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>Hup</td>
<td>Mouse (i.v.)</td>
<td>279</td>
</tr>
<tr>
<td>Neisseria meningitidis</td>
<td>HmbR</td>
<td>Rat (i.p.)</td>
<td>280</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Isd</td>
<td>Mouse (i.v.)</td>
<td>281</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>Shr</td>
<td>Zebradish (i.m.)</td>
<td>282</td>
</tr>
<tr>
<td>Vibrio vulnificus</td>
<td>Hup</td>
<td>Mouse (i.p.)</td>
<td>282</td>
</tr>
</tbody>
</table>

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a i.t., intratracheal; i.n., intranasal; t.u., transurethral; i.p., intraperitoneal; i.v., intravenous; i.m., intramuscular.

b Due to space limitations, we cite only one reference even though there are multiple studies published in many cases.
(HasASM) and *P. aeruginosa* (HasAPA) have been structurally characterized and are nearly identical globular monomeric proteins with a unique αβ fold, capable of coordinating a single molecule of ferric heme with very high affinity (HasASM; $K_d = 1.8 \times 10^{-11}$ M) (36). Common to substrates of type I secretion systems, these hemophores possess a C-terminal export motif and are transported to the external milieu by a dedicated ATP-binding cassette (ABC) exporter (37). Upon secretion, HasA-type hemophores are capable of acquiring heme from a broad range of host hemoproteins including hemoglobin, hemopexin, and myoglobin (38), but the means by which HasA facilitates heme acquisition is currently a matter of debate. The broad substrate range of HasA, and its ability to nondiscriminately acquire heme from hemoglobin of unrelated organisms (e.g., human and bovine, leghemoglobin from leguminous plants) (32, 38), supports the notion that HasA acquires heme without direct interaction with these hemoproteins (38).

Upon binding at the cell surface, holo-HasA is not internalized but, rather, transfers heme to an iron-regulated, outer membrane receptor protein for import (HasR) (Fig. 2) (39, 40). The hemophore is known to complex tightly with HasR, although the mechanism of heme transfer between the two is not yet fully understood (41, 42). HasR binds heme with lower affinity than HasA (39), and the binding of holo-HasA to the receptor is thought to induce conformational changes that successively weaken the binding of heme to HasA (43). Heme binding by HasR is mediated by two highly conserved histidine residues (44), found in numerous other outer membrane heme receptor proteins including HemR of *Y. enterocolitica* (45), ShuA of *Shigella dysenteriae* (46), and HmuR of *P. gingivalis* (47). Notably, HasR can independently mediate the uptake of free and hemoglobin-bound heme, but HasR and HasA function synergistically to enhance heme uptake by approximately 100-fold over the outer membrane receptor alone (48).

HasR is a member of the TonB-dependent transporter (TBDT) family (Fig. 3). The uptake of heme by TBDTs is an energetic process driven by TonB and the proton motive force or, in the case of the *has* system of *S. marcescens*, the HasR-specific TonB ortholog, HasB (49, 50). In addition to the expected negative regulation by iron and Fur, expression of the *has* operon is

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**FIGURE 2** Model of iron uptake mechanisms in Gram-negative and Gram-positive bacteria. Diagrams depicting the envelope proteins required for the uptake of iron, or iron scavenged from siderophores, heme, or transferrin. This is a composite diagram and represents mechanisms used by many pathogenic bacteria, as described in the text. OM, outer membrane; PG, peptidoglycan; CM, cytoplasmic membrane; sid, Fell-siderophore; Hm, heme; Tf, transferrin; OMP, outer membrane porin; HO, heme oxygenase; Hb, hemoglobin; Hp, haptoglobin. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (274), copyright 2012.
further modulated by heme and extracytoplasmic function sigma (σ) and anti-σ factors (HasI and HasS, respectively), through a signal cascade induced by the binding of heme-loaded HasA to HasR (51–53). Despite the known regulation of bas by heme, it is not known how expression of this hemophore-based heme acquisition system influences the in vivo survival and virulence of pathogens in which it is employed. Indeed, to date, the role of bas has been assessed solely in a murine model of bubonic plague, where it was shown that neither bas nor the hemin uptake system, bmu (discussed below), are required for the pathogenicity of Y. pestis under the conditions assessed (54).

**HxuA, a Gram-negative pseudo-hemophore**

Restricted to members of the *Pasteurellaceae* and characterized only in *H. influenzae* type b (Hib) and unencapsulated nontypeable *H. influenzae* (NTHi) isolates, the heme-hemopexin utilization protein, HxuA, contrasts with HasA-type hemophores in its extremely limited range of substrate specificity and its means of facilitating heme acquisition (33, 55, 56). HxuA bears an

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**FIGURE 3** Model of TonB-dependent transport in Gram-negative bacteria. An iron-siderophore complex (blue hexagon) entering through a TonB-dependent transporter (TBDT) in the outer membrane (OM). Although the transport of an iron siderophore complex is shown here, iron, or other iron complexes, use similar uptake mechanisms (e.g., Fell, heme) (see Fig. 1). Movement through the TBDT requires an interaction of the TonB box (located near the N-terminus of the TBDT sequence) with the TonB protein, with the energy for conformational changes provided by the proton motive force captured by the ExbB and ExbD proteins. Once in the periplasm, the iron-loaded siderophore complex is recognized by a substrate-binding protein which delivers the complex to an ABC transporter in the cytoplasmic membrane (CM). Depending on the particular system, iron is released from the siderophore in the cytoplasm by either destruction of the siderophore or reduction on the metal (as shown). Intracellular iron, via Fur, negatively regulates transcription of genes encoding high-affinity iron acquisition systems. In some TBDTs, an N-terminal extension is present to provide an extra layer of control of gene expression, in addition to Fur. This involves an anti-σ factor and extracytoplasmic function σ-factor, allowing for gene expression in response to the uptake of particular iron chelates. Modified with permission from Annual Review of Microbiology, volume 64 © by Annual Reviews, http://www.annualreviews.org. See Noinaj et al. (91).
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N-terminal signal peptide and belongs to a two-partner secretion system, where export of the “pseudohemopore” is mediated by the outer membrane partner protein, HxuB (57, 58). Depending upon the strain, HxuA is either anchored to the outer membrane or at least partially released into the supernatant, whereupon it facilitates the release of heme solely from heme-hemopexin complexes (59). Although the exact mechanism by which HxuA liberates heme from heme-hemopexin is unknown, it differs from HasA in that direct protein-protein interactions are required (56, 60). Further, by strict definition, HxuA is not a hemophore, because it lacks a specific heme-binding domain. Instead, HxuA appears to release heme from heme-hemopexin for capture by HxuC, a TBDT (60). Similar to HasR, HxuC is capable of mediating heme uptake independently of HxuA and is additionally capable of acquiring heme from serum albumin and hemoglobin (61, 62). The role of hxA and hxuB in H. influenzae heme-iron acquisition should not be overlooked, however, because both are essential to the utilization of heme-hemopexin by this heme auxotroph (57). Further, the Hxu-encoding locus, hxuCBA, has been implicated in H. influenzae pathogenicity, because strains lacking this operon are less virulent in an infant rat model of hematogenous meningitis (61). Interestingly, the expression of HxuA, as well as transferrin-binding proteins A and B (TbpA and TbpB, respectively), has been detected in middle ear aspirates of children afflicted with otitis media (63), although further investigation is required to fully elucidate the role of hxuCBA in the utilization of heme-hemopexin by H. influenzae during infection.

NEAT domains and NEAT-containing Gram-positive hemophores

The hemophores of Bacillus anthracis, IsdX1 and IsdX2, are components of an iron-regulated surface determinant (Isd) pathway (64, 65) that includes cell surface-associated proteins involved in the acquisition and transfer of heme-iron through the Gram-positive cell wall. Heme coordination by IsdX1 and IsdX2 is mediated by NEAT domains, of which IsdX1 has one and IsdX2 has five (64, 65). NEAT domains were first identified in genetic loci encoding for putative ferric iron transporters (66), although it has since been revealed that these diverse ~120-amino-acid-residue sequences actually encode for discrete modules involved in heme and/or hemoprotein binding, heme extraction, and NEAT: NEAT-mediated heme transfer (67–69). NEAT domains are found in both pathogenic and nonpathogenic members of the phylum Firmicutes and, while lacking conformity in their amino acid sequences, are highly conserved at a structural level. A prototypical NEAT domain is comprised of eight β-strands and a small α-helix, which together fold to form a hydrophobic pocket in which heme may be bound (67). Heme coordination by a NEAT domain is mediated by a conserved YXXXXY motif, where preservation of the first tyrosine residue reliably predicts heme-binding functionality (70). The mechanisms of hemoprotein-binding and heme extraction by NEAT domains appear to differ between organisms but have been most extensively explored for the Isd system of S. aureus (see below).

Each NEAT domain of IsdX1 and IsdX2 of B. anthracis has been functionally characterized, and together, both hemophores are capable of liberating heme from hemoglobin, binding heme through NEAT domains possessing the aforementioned YXXXXY motif, and transferring extracted heme to the cell wall-associated heme-binding protein, IsdC, for eventual uptake (64, 65, 71, 72). Unlike the heme-extracting Isd proteins of S. aureus (IsdB and IsdH), where NEAT domains function synergistically in extracting heme from hemoglobin, the sole NEAT domain of IsdX1 appears to independently fulfill this task (65). Further, IsdX1 is capable of transferring heme to IsdX2 and, while heme transfer between two hemophores may seem counterintuitive, it has been suggested that the multiple heme-binding domains of IsdX2 may serve to sequester heme when present in excess and thus limit the rate of uptake (71, 72). Alternatively, given that IsdX2 is partially cell wall-associated, it is possible that this hemophore doubles as a surface receptor for heme.

Encoded from an otherwise uncharacterized Isd-like locus, L. monocytogenes expresses two NEAT-domain-containing, heme-extracting proteins: hemin-binding proteins 1 and 2 (Hbp1 and Hbp2), respectively (12, 73). Apart from the hemophores of B. anthracis, Hbp2 is the only other hemophore conclusively identified in the Gram-positive pathogens. While Hbp1 is thought to be cell wall–associated, Hbp2 is both secreted as a true hemophore and, to a lesser extent, anchored to the cell surface (12, 73). In the current model, heme is extracted from hemoglobin or captured from the external milieu by Hbp2, using a unique coordination method not involving the YXXXXY motif, and is subsequently transferred to surface-associated Hbp2 or Hbp1 for uptake (73). Hbp1 and Hbp2 both have a high affinity for heme and hemoglobin, suggesting that, as with other Isd-like systems, they are advantageous at physiological concentrations (nM range) (12, 73). The means by which heme
delivered by Hbp2 is transported through the listerial cell envelope is not known, but likely it occurs in a manner analogous to other Isd-containing bacteria and/or may involve the uncharacterized heme/hemoglobin-uptake ABC transporter, HupDGC (73).

HmuY and the gingipain proteases: a collaborative system for heme acquisition in \textit{P. gingivalis}

Iron acquisition by the Gram-negative, oral anaerobe \textit{P. gingivalis} is a fascinating and complex example of how pathogens have evolved unique iron uptake mechanisms central to their distinct pathophysiology. \textit{P. gingivalis} is a key etiological agent of periodontitis, a disease characterized by inflammation, bleeding, and degradation of the tissues supporting teeth. The destructive process initiated by \textit{P. gingivalis} leads to the formation of periodontal pockets suffused with an inflammatory exudate containing iron complexes including transferrin, serum albumin, hemoglobin, haptoglobin, and heme-hemopexin (74). \textit{P. gingivalis}, despite lacking endogenous siderophores, grows robustly within these pockets due to a versatile means of accessing heme (75). Indeed, heme is essential to the virulence of \textit{P. gingivalis}, not only because it is incapable of synthesizing protoporphyrin IX (PPIX), but also because heme is a fundamental component of the black pigment employed by the bacterium, purportedly to protect against peroxide stress (76). Heme acquisition in \textit{P. gingivalis} is distinctively aided by the secretion of arginine- and lysine-specific cysteine proteases (RgpA and RgpB, and Kgp, respectively), collectively referred to as “gingipains.” Additionally, these gingipains contribute to the inflammation and tissue destruction characteristic of periodontitis, promote adherence to epithelial cells, and assist in multispecies biofilm development and thus, are multifactorial virulence determinants of \textit{P. gingivalis} (77). Both RgpA and Kgp are multidomain proteins consisting of an N-terminal proteolytic domain and C-terminal hemagglutinin domain.

In a cooperative fashion, gingipains act first to oxidize heme within hemoglobin through RgpA, which functions both to reduce the affinity of hemoglobin for bound heme and to render the resulting methemoglobin more susceptible to proteolysis by Kgp (78, 79). Heme released through this sequential proteolytic degradation is then rapidly sequestered by the lipoprotein hemophore, HmuY (for heme utilization), and is transported into the periplasm by the cognate TBDT, HmuR (80). HmuY is found both attached to the outer membrane and released to the external milieu as a classical hemophore, where it is proposed that Kgp is responsible for cleaving the protein from the surface of the cell (34). HmuY is additionally capable of independently capturing free heme and removing heme directly from methemoglobin and methemalbumin (81), thus highlighting its utility in accessing heme sources commonly encountered by the pathogen. Further, the gingipains nonspecifically degrade other host iron-containing proteins, including haptoglobin, hemopexin, transferrin, and albumin, as a source of both iron and peptides (82), suggesting that together with the HmuY hemophore, these proteases play a crucial role in the survival and virulence of \textit{P. gingivalis} within its discrete host niche. The mechanism for translocation of heme into the cytosol of \textit{P. gingivalis} is not known, but it is proposed that genes within the \textit{hmuYRSTUV} locus encode for a heme-specific ABC-type transporter (83).

Other secreted proteases as putative liberators of heme

As demonstrated for the gingipain proteases of \textit{P. gingivalis}, the secretion of proteolytic enzymes capable of degrading host heme-iron complexes would be a valuable asset to pathogens, particularly those lacking dedicated systems for the acquisition of iron or heme from these substrates. The prevalence of secreted hemoprotein proteases may perhaps be underappreciated and, outside of \textit{P. gingivalis}, have only been marginally characterized in pathogenic strains of \textit{E. coli}. As members of the diverse serine protease autotransporter of \textit{Enterobacteriaceae} (SPATE) family, these enzymes possess an N-terminal leader peptide that targets them to the periplasm via type V secretion, a functional passenger domain carrying a serine protease motif, and a C-terminal translocator domain, which forms a β-barrel in the outer membrane and allows for external release of the passenger domain through proteolytic cleavage (84). The passenger domain of hemoglobin protease and enteropathogenic \textit{E. coli} secreted protein C (EspC) is thought to facilitate both hemoglobin degradation and heme-binding in enteropathogenic \textit{E. coli} (85, 86), suggesting that they function like hemophores. The observed \textit{in vitro} activity of hemoglobin protease and EspC in heme-iron extraction, however, has not been confirmed, nor has a TBDT that interacts with hemoglobin protease or EspC been identified. The expression of hemoglobin protease by \textit{E. coli} has been implicated, however, in the development of cospecies intra-abdominal abscesses with \textit{Bacteroides fragilis}, a heme auxotroph, through what is thought to be pathogenic synergy; \textit{B. fragilis} provides fibrin deposition that facilitates abscess development and immune evasion, whereas \textit{E. coli}...
liberates heme from hemoglobin via hemoglobin protease for utilization by both bacteria (87). The exploitation of heme liberated from host complexes by heterologous microorganisms is a potentially important facet of polymicrobial infections.

Heme Capture at the Cell Surface
A universal mechanism for iron transport through the Gram-negative cell envelope
The outer membrane in Gram-negative bacteria serves as a coarse selective barrier, restricting potentially toxic compounds, such as antibiotics, from unfettered access to the periplasmic space. Porins allow for the nonspecific diffusion of small (<600 kDa), hydrophilic molecules and ions, including Fe^{2+} (Fig. 2), through the outer membrane, while the uptake of some nutrients, including disaccharides, nucleosides, and phosphates, occurs through facilitated diffusion (88). In contrast, the uptake of larger molecules and/or those present at exceedingly low concentrations in the extracellular milieu requires dedicated high-affinity, energy-dependent transporters to traverse the outer membrane (88, 89). Iron complexes, such as heme, transferrin, and most siderophores, exceed the size exclusion criteria for unimpeded diffusion across the outer membrane, and thus, the uptake of iron from these substrates is invariably facilitated by iron-regulated, TonB-dependent outer membrane receptors (Fig. 2 and Fig. 3).

Structure, function, and regulation of TonB-dependent transporters
As discussed above for hemophores, specific outer membrane receptors in Gram-negative bacteria serve as the recipients of heme, either through transfer from hemophores or directly from the external environment, whereupon they facilitate the transport of heme, intact, into the periplasm. Acquisition of iron from transferrin and lactoferrin is similarly mediated by TBDTs that directly bind these host glycoproteins or bind siderophores that have stripped iron from them. While the vast majority of TBDTs are involved in the acquisition of iron, other substrates such as vitamin B_{12}, nickel complexes, colicins, phages, and carbohydrates may also gain entry to the periplasmic space through these transporters (90). To date, approximately 50 TBDT structures have been solved, alone and in complex with various ligands, and each consists of a transmembrane-spanning β-barrel encircling an independently folded N-terminal plug domain (see Fig. 4). Substrate-binding residues are located on the exofacial side of the plug, lining the interior of the pore, and on extracellular loops of the β-barrel and are
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customized to accommodate binding of the preferred substrate (91). The N-terminus of the pore domain also contains a weakly conserved motif of approximately seven amino acid residues, referred to as the TonB-box, which are responsible for interacting with the TonB protein that is part of the TonB-ExbB-ExbD complex, as described below.

In addition to the plug and barrel domains of canonical TBDTs, a subset of these proteins, including HasR of *S. marcescens* (53), the ferric citrate receptor, FecA, of *E. coli* (92), and the ferric pyoverdine receptor, FpvA, of *P. aeruginosa* (93), possess a third domain, localized within the periplasm and designated the “N-terminal extension” or “signaling domain” (Fig. 3) (94–96). TBDTs bearing such a domain not only are subject to regulation by Fur, but are also capable of fine-tuning their own expression through a α/anti-α-factor signaling cascade induced upon binding of the iron-loaded substrate to the transporter. Briefly, and in general, an anti-α-factor localized to the inner membrane sequesters an associated and often cotranscribed extracytoplasmic function α-factor under nonactivating conditions (i.e., in the absence of substrate). In the presence of a substrate, the N-terminal extension of the TBDT interacts with the associated anti-α-factor, promoting release of the once-bound α-factor into the cytosol. The extracytoplasmic function α-factor in turn directs RNA polymerase to transcribe the operon encoding the transporter and any accompanying genes involved in substrate acquisition (e.g., hemophore or siderophore biosynthesis genes). In this manner, a signal can be relayed from the cell surface to the cytoplasm indicating the availability of substrates for acquisition, and the bacterium can respond with commensurate expression of the cognate TBDT for uptake.

The outer membrane of Gram-negative bacteria lacks an energy source, and thus the proton motive force of the cytoplasmic membrane is required to fuel the active transport of iron complexes into the periplasm. Transduction of energy to the outer membrane is mediated by the inner membrane complex TonB-ExbB-ExbD (97), through a mechanism which is not yet fully understood and the details of which are outside the scope of this chapter, although several comprehensive reviews on the subject are available (89, 91, 98, 99). In brief, TonB bears three functional domains: an N-terminal signal sequence that targets the protein for translocation to the cytoplasmic membrane, a proline-rich periplasmic spacer region thought to confer flexibility to TonB and allowing it to span the periplasmic space, and a C-terminal domain which interacts with TonB-boxes of TBDTs. Binding of an iron complex to its respective TBDT promotes unfolding of the TonB box, which signals to TonB-ExbB-ExbD that a substrate is bound and ready for transport. Although the exact nature of the signaling between the TonB-box and TonB-ExbB-ExbD is unknown, it induces the TonB-box to physically engage TonB (Fig. 3) (100, 101), energizing the transporter for uptake of the bound iron complex. While it is commonly acknowledged that the plug domain, which otherwise occludes the β-barrel pore, must then undergo a conformational change to allow for passage of the substrate into the periplasm and likely involves at least partial expulsion of the plug from the pore (102), the nature of this domain rearrangement has yet to be fully characterized and indeed remains a point of contention (91, 103).

**ABC transporters for heme transport across the inner membrane**

Once in the periplasm, heme must subsequently be transported across the Gram-negative inner membrane and into the cytosol, a process mediated by ABC-type transporters. Notably, while this discussion specifically pertains to heme transport in Gram-negative bacteria, transport occurs analogously in Gram-positive bacteria across the cytoplasmic membrane and is similarly applicable to the uptake of other iron substrates, including siderophores and Fe$^{3+}$ (Fig. 2). As depicted in Fig. 5, canonical prokaryotic ABC-type transporters consist of three central components: (i) a dedicated high-affinity substrate-binding protein (SBP), which captures and shuttles the substrate through the periplasmic space in Gram-negative bacteria or is lipid-anchored to the cytoplasmic membrane and captures heme from the external milieu in Gram-positive bacteria, (ii) an associated permease, usually comprised of two integral transmembrane protein subunits, and (iii) two peripheral ATPases, which power substrate translocation via ATP hydrolysis (104). In Gram-negative bacteria, the periplasm is a dynamic and congested space, and thus SBPs play an essential role in the trafficking of scantily available substrates, such as heme, to the inner membrane. Further, the specificity of an ABC-type transporter is usually governed by the ligand(s) bound by its cognate SBP. To date, over 50 heme-specific SBPs have been putatively identified (105), although few have been structurally and functionally characterized.

The vast majority of SBPs involved in the coordination of inorganic iron and organic iron complexes, such as heme, siderophores, and cobalamin, are categorized as cluster A, class III SBPs (106). These proteins are found exclusively in association with ABC
transporters and possess two independently folded domains, each comprised of a central $\beta$-sheet surrounded by $\alpha$-helices, that are joined by an $\alpha$-helical linker of approximately 20 amino acid residues (Fig. 5). The substrate (e.g., heme) is coordinated in an interdomain cleft and, in class III SBPs, binding involves minimal conformational changes to the protein, owing to inflexibility of the $\alpha$-helical spine. Docking of an SBP with its cognate ABC transporter is facilitated by salt bridges formed between highly conserved glutamic acid residues located at the apex of each SBP lobe and positively charged patches of arginine and/or lysine residues on the periplasmic face of the permease subunits. The formation of this stable SBP:ABC transporter complex, as illustrated in the paradigmatic example of the *E. coli* vitamin B$_{12}$-specific SBP, BtuF, and its associated transporter, BtuCD (Fig. 5) (107), is necessary to induce translocation of the substrate.

The first functionally characterized prokaryotic heme ABC transport system, encoded by the hemin uptake locus, hemRSTUV, of *Y. enterocolitica* is comprised of the heme-specific periplasmic SBP HemT, the integral transmembrane protein HemU, and the ATP-binding protein HemV (108). In total, this locus encodes for a complete heme uptake system in *Y. enterocolitica*, including a TBDT capable of independently binding and extracting heme from host hemoproteins at the cell surface (HemR) (109), and a cytosolic heme-carrier protein which sequesters free heme upon transport into the cell (HemS) (110). HemTUV-homologous heme ABC transporters have been functionally interrogated in a number of Gram-negative pathogens, including HmuTUV of *Y. pestis* (111, 112), ShuTUV of *Shigella dysenteriae* (113), HutBCD of *V. cholerae* (114), and PhuTUV of *P. aeruginosa* (115), and in each case, the transporter is required for efficient heme transport into the cytosol. Structural elucidation of both the apo- and holo-forms of the heme-binding SBPs ShuT and PhuT confirmed that both proteins exhibit classical class III SBP architecture, where heme is coordinated in a deep but narrow groove between the globular N- and C-terminal domains with little domain rearrangement upon binding. Although the binding pockets of the two proteins differ substantially, in both ShuT and PhuT heme is pentacoordinate, with a conserved tyrosine residue located within the N-terminal domain of the SBP serving as the heme axial ligand (116). Indeed, despite poor overall primary sequence homology, nearly every putative periplasmic heme-specific SBP identified to date appears to possess such a tyrosine residue, suggesting a common mechanism for heme binding in these proteins (105). Obviously, exceptions exist, and HmuT of *Y. pestis* appears to represent a unique subset of heme-specific SBPs, where two stacked heme molecules are coordinated in a single, comparatively large, binding cleft by a tyrosine and a histidine residue. The presence of these two residues may predict dual heme binding in other SBPs, and it has been proposed that in these systems, the two heme molecules are transported simultaneously (117).

Structural and functional characterization of the representative ABC transporters BtuCDF of *E. coli* and HmuTUV of *Y. pestis* suggests that the mechanism of substrate translocation (vitamin B$_{12}$ and heme, respectively) is conserved (118, 119). In brief, it appears that in the absence of a loaded SBP and ATP, the transporter is closed to the cytoplasm but open to the periplasm, forming an outward-facing cavity large enough to accommodate most of the substrate. The binding of the

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**FIGURE 5** Structure of *E. coli* BtuCDF. A ribbon diagram of the *E. coli* BtuCDF complex (PDB 2QI9), representative of the iron-siderophore/cobalamin family of cytoplasmic membrane transporters. The two lobes of the substrate-binding protein BtuF (magenta) are docked on top of the two permease domains (BtuC, monomers colored yellow and green) which are associated with ATP-binding proteins (BtuD, monomers colored red and blue).
The iron-regulated surface determinant pathway: a Gram-positive heme acquisition mechanism

While not possessing an outer membrane through which heme must be actively transported, the thick peptidoglycan layer (∼20 to 40 nm) of Gram-positive bacteria impedes the accessibility of heme to cell membrane–associated receptor proteins. This impediment necessitates a means by which heme can be conveyed through the cell wall, particularly when the concentration of available extracellular heme is low (<50 nM range) (12, 123, 124). Indeed, mechanisms that shuttle heme from the cell surface to the cytoplasmic membrane have been described in a number of Gram-positive pathogens including S. aureus, Staphylococcus lugdunensis, B. anthracis, L. monocytogenes, Streptococcus pyogenes, and C. diphtheriae (for a recent comprehensive review see reference 125). As introduced above, the Isd pathway is the best characterized of these mechanisms, has been most extensively investigated in S. aureus (see Fig. 2), and herein is discussed as a paradigmatic example of such heme transfer systems in the Gram-positive pathogens.

Unlike B. anthracis and L. monocytogenes, S. aureus is not known to elaborate hemophores, and thus heme extraction, binding, and transfer are all performed by cell surface–associated proteins. In its entirety, the Isd pathway of S. aureus consists of nine iron-regulated proteins, IsdA through IsdI, and a genetically linked sortase B enzyme (SrtB) (126, 127). Three of these proteins (IsdA, IsdB, and IsdH) are anchored to the cell wall by sortase A (127), where the latter two proteins bind and extract heme from hemoproteins at the cell surface (124, 128, 129). IsdB and IsdH transfer freed heme to IsdA or directly to a fourth cell wall–associated heme-binding protein, IsdC (130, 131). IsdC is positioned deep within the cell wall by SrtB (126), and this localization is purportedly ideal for funneling heme from IsdA, IsdB, and/or IsdH to the membrane-associated protein, IsdE (130–134). Heme transfer through the cell wall is a passive process that is driven, in part, by the successive increase in affinity that each Isd protein has for heme, relative to the preceding protein (IsdB/IsdH < IsdA < IsdC < IsdE) (132–135). Transient, ultraweak (≥ micromolar affinity), “handclasp”–type interactions facilitate the movement of heme specifically between the NEAT domains of interacting Isd proteins (130, 136–138). IsdE does not contain a NEAT domain, and instead is a type III SBP, which along with its cognate membrane permease, IsdF, is a member of the ABC transporter superfamily (133, 134). The requisite ATPase for energizing the transfer of heme across the cytoplasmic membrane by IsdEF in S. aureus has yet to be identified.

The Isd pathway in S. aureus is required for uptake of iron from physiological concentrations of heme or hemoglobin (124), and indeed IsdA, IsdB, IsdC, IsdH, and the heme-degrading enzymes IsdG and IsdI are all required for maximal virulence of S. aureus in murine models of infection (124, 128, 139–141). As with the hemophores of Gram-positive pathogens, NEAT domains, ranging from one in each IsdA and IsdC, to two in IsdB, and three in IsdH, are the functional units involved in heme–iron acquisition in S. aureus (70, 136, 142–145). Each of the staphylococcal NEAT domains bears distinct specificities, where the N-terminal NEAT domains of IsdB (IsdB₁₁) and IsdH (IsdH₁₁ and IsdH₃₂) bind hemoglobin (or hemoglobin–haptenoglobin) (129, 146–150), and the C-terminal NEAT domains of these proteins (IsdB₃₂ and IsdH₃₃), as well as those of IsdA and IsdC, bind heme (70, 136, 143, 151). Unlike the sole NEAT domain of IsdX1, which is independently capable of mediating heme extraction from hemoglobin, the individual NEAT domains of IsdB and IsdH appear to function cooperatively as a conserved unit to fulfill this purpose. In brief, this extraction module is comprised of an N-terminal hemoglobin-binding domain (IsdB₁₁ or IsdH₁₁), an intervening and inflexible α-helical linker region, and a contiguous C-terminal holo-SBP together with ATP permits release of the substrate into the lumen of the transporter, which undergoes a conformational change, trapping the substrate within a centrally located cavity. Upon ATP hydrolysis, the substrate is effectively expelled from the lumen into the cytosol, and the transporter collapses, likely to avoid leakage of cytoplasmic contents. The mechanism by which the apo-SBP is released back into the periplasm and the transporter is reset is currently unknown but likely involves ATP hydrolysis (For a comprehensive review of ABC transporters see reference 120). The potential toxicity of heme necessitates that it be rapidly sequestered or degraded upon transport into the cytosol and, notably, many loci encoding heme ABC transporters also include genes expressing cytosolic heme-binding proteins. Such heme carrier proteins have been characterized in S. dysenteriae (ShuS), P. aeruginosa (PhuS), and Y. enterocolitica (HemS), where heme is received directly from the associated ABC transporter at the cytoplasmic membrane (110, 121, 122) and ostensibly is shuttled safely to either heme storage or degradation pathways within the cell.
heme-binding domain (IsdBN2 or IsdHN3) (148, 150, 152–154). Working in concert with the hemoglobin-binding domain, the indispensable linker region is proposed to induce steric strain on hemoglobin, thereby facilitating the dissociation of heme (148, 150, 153), while at the same time positioning the associated heme-binding domain in close proximity to the globin heme-binding pocket (148). Upon release from hemoglobin, heme is rapidly seized by the C-terminal heme-binding domain of IsdB or IsdH for subsequent transfer to IsdA or IsdC, prior to transport into the cytoplasm for use by the bacterium (130, 131).

The Isd pathway has been sparsely characterized outside of S. aureus. Aside from atypically pathogenic S. lugdunensis (155–157), coagulase-negative staphylococci do not encode for an Isd system. L. monocytogenes appears to encode an isd-like locus for uptake of heme from Hbp1 and Hbp2, but the functionality of this system has not yet been explored (12, 73). In B. anthracis, IsdC performs a central role in receiving heme from IsdX1 and IsdX2, but homologs of other cell-surface-associated Isd proteins are missing in this organism (65, 158). Uptake of heme by B. anthracis hemophores may be aided by an alternative NEAT-domain-containing protein, BslK (for B. anthracis S-layer homology protein K), which has been proposed to facilitate heme transfer through the S-layer crystalline array of this pathogen to IsdC (70, 159). Notably, mutation of none of the Isd systems characterized to date fully abrogates growth on heme/hemoglobin as an iron source, except at low nM heme/hemoglobin concentrations, suggesting that alternative mechanisms for heme-iron acquisition likely exist in the Gram-positive pathogens.

**Alternative Mechanisms for Heme Acquisition in Gram-Positive Pathogens**

An extensive review of all heme-iron acquisition systems in bacterial pathogens is outside the scope of this chapter, but some of the known mechanisms are summarized in Table 1 (for an in-depth discussion, see reference 125). Two notable non-Isd heme shuttle pathways have been identified in the Gram-positive pathogens: the streptococcal iron acquisition system of S. pyogenes and the hemin-uptake and heme transport-associated locus (Hmu-Hta) of C. diphtheriae (160, 161). The streptococcal iron acquisition system functions analogously to the Isd system of S. aureus and appears to include divergent members of the NEAT family of proteins, including the streptococcal hemoprotein receptor (Shr; an IsdB/IsdH analog), and the streptococcal heme-associated cell surface protein (Shp; an IsdC analog) (160, 162). Hemoprotein binding by Shr, however, does not occur by way of the NEAT domain, but rather by a unique N-terminal domain (162). Heme extracted from host hemoproteins by Shr, using the composite N-terminal–NEAT domain, is transferred via Shp to a heme ABC transporter (HtsABC; an IsdEF homolog) for uptake (160, 162, 163). Notably, Shr is both highly immunogenic and promotes the survival of S. pyogenes in whole human blood and thus has been investigated as a vaccine target (164).

In contrast, the Hmu-Hta system of C. diphtheriae is distinct from heme acquisition systems in other Gram-positive pathogens. Hmu-Hta is comprised of six DtxR-regulated genes: three that encode an ABC transporter bearing homology to hmuTUV of Y. pestis (161), two that encode heme-binding cell-surface-associated proteins (HtaA and HtaB), and one protein of unknown function (HtaC) (165). Similar to Shr, HtaA and HtaB are not anchored to the cell wall by sortases but, rather, possess a C-terminal transmembrane domain for attachment (165, 166). Further, these two proteins lack NEAT domains and instead mediate heme extraction and binding using a unique conserved region of ~150 amino acid residues. HtaA appears to facilitate heme uptake from host hemoproteins, whereas HtaB is proposed to function as an intermediary in transferring heme to HmuTUV (165–167). Heme uptake by C. diphtheriae may additionally be aided by the corynebacterial heme transport-associated proteins (ChtA, ChtB, and ChtC), but further efforts are required to elucidate the means by which these systems cooperatively promote heme-iron acquisition in this pathogen (168).

**The Fate of Intracellular Heme**

Heme that is not incorporated as a prosthetic group into bacterial enzymes must be sequestered, degraded, or effluxed to guard against toxicity. As previously discussed, the import of heme in some bacteria is coupled with its rapid seizure by heme-carrier molecules such as ShuS, PhuS, and HemS of S. dysenteriae, P. aeruginosa, and Y. enterocolitica, respectively, and homologs of these proteins are found widely dispersed among the Proteobacteria. Heme-degrading enzymes are required by eukaryotes and prokaryotes alike to liberate iron from the porphyrin ring.

Until recently, heme degradation in bacteria was thought to occur solely through the action of heme oxygenases (HOs) that function analogously to eukaryotic HOs and catalyze the conversion of heme to biliverdin with the release of iron and CO (169). Indeed, such canonical HOs have been identified in both Gram-positive
and Gram-negative pathogens including HmuO of *C. diphtheriae* (170, 171) and HemO of *Neisseria* spp. (172) and *P. aeruginosa* (173). A novel class of heme-degrading enzymes was recently identified in Gram-positive pathogens including *S. aureus*, *S. lugdunensis*, *Bacillus* spp., and *M. tuberculosis* (174). For a detailed discussion on heme degradation by both canonical and noncanonical HOs, readers are referred to recent comprehensive reviews by Wilks and colleagues (175, 176).

Several mechanisms exist for guarding microbes against the toxicity associated with excess intracellular heme and include inhibiting uptake or endogenous synthesis through Fur or DtxR-mediated repression, sequestration in heme-binding proteins, degradation by canonical and noncanonical HOs, and efflux. Heme is generally more toxic to Gram-positive pathogens than to Gram-negative pathogens, and although the reasons for this are unclear, mechanisms for heme efflux have almost exclusively been identified in Gram-positive organisms. Heme toxicity and efflux will not be discussed in detail here, and we refer the reader to an excellent review on the subject (177).

**ACCESSING TRANSFERRIN-IRON**

An important facet of innate immunity, and indeed one of the first lines of defense against invading bacterial pathogens, is the low availability of iron at host mucosal surfaces and within the serum and lymph, owing to sequestration by lactoferrin and transferrin, respectively. As with the utilization of heme-iron complexes, many successful pathogens are capable of circumventing iron withholding by transferrin and lactoferrin, by usurping iron sequestered by these host glycoproteins. Three main mechanisms exist for the utilization of transferrin and/or lactoferrin by bacteria: the elaboration of cell surface–associated transferrin- or lactoferrin-specific binding proteins (Tbps and Lbps, respectively), the secretion of small iron-chelating molecules, referred to as siderophores, and the extracellular release of broad substrate range reductases, which reduce ferric iron to ferrous iron for subsequent uptake by inorganic iron permeases. All three mechanisms ultimately function to liberate iron from transferrin and/or lactoferrin prior to its transport into the cell (see Tables 2 and 3).

**Transferrin- and Lactoferrin-Binding Proteins: the TbpA/TbpB Paradigm**

The establishment of infection by pathogenic *Neissericaceae, Pasteurellaceae*, and *Moraxellaceae* species unequivocally begins with colonization of a specific mucosal surface such as the urogenital tract (*Neisseria gonorrhoeae*) or the upper respiratory tract (*Neisseria meningitidis, Moraxella catarrhalis*, and *H. influenzae*). Iron available to mucosal pathogens exists predominantly in the extracellular state and, as such, survival and proliferation of the aforementioned bacteria are undoubtedly aided by their capacity to exploit host transferrin and/or lactoferrin directly as iron sources. Unlike most Gram-negative pathogens, the ability of these microbes to utilize iron-containing host glycoproteins is not facilitated by the production of siderophores (as discussed below) but instead is mediated by highly specific, cell surface–associated transferrin- or lactoferrin-binding proteins (178–180).

The paradigmatic transferrin-receptor in Gram-negative bacteria is a Fur-regulated bipartite system consisting of an outer membrane receptor protein, TbpA (181), and a surface-associated lipoprotein, TbpB (182) (Fig. 2). Both TbpA and TbpB bind transferrin, but TbpA nondiscriminantly binds both apo- and holo-transferrin, whereas TbpB selectively binds holo-transferrin (183). TbpB purportedly functions as bait to capture iron-loaded transferrin from the extracellular milieu and deliver it to TbpA for TonB-dependent uptake. Similar to the mechanism of heme uptake by HasA/HasR, TbpA is capable of independently mediating transferrin-iron acquisition (182, 184), and the presence of TbpB substantially enhances the efficiency of transferrin-iron acquisition by the bacterium.

In contrast to heme and siderophores, which are transported intact through the outer membrane, iron must first be liberated from transferrin prior to uptake by TbpA. Both TbpA and TbpB of the human pathogen *N. meningitidis* exclusively bind the C-lobe of human transferrin (hTf) (185, 186), through nonoverlapping binding sites (186, 187). As depicted in Fig. 6, TbpB is a bilobate protein, where each lobe is comprised of an eight-stranded β-barrel and an adjacent four-stranded “handle” domain (187). The C-lobe of holo-hTf effectively docks onto the “cap” of the N-lobe of TbpB with minimal conformational change to either protein, and the two are held together by extensive interactions along a large binding interface (for details see reference 187). The structural characterization of TbpB in complex with hTf has provided insight into both the specificity of TbpB for transferrin solely of its host species and its role in iron acquisition from transferrin. First, TbpB interacts with a region of hTf (loop L496–515) that is highly variable in sequence and length between mammalian transferrin homologs, where variations within the TbpB recognition site sterically hinder binding of the protein
and help to explain the lack of cross-species transferrin-iron acquisition by TbpB (187). Second, instead of helping to liberate ferric iron from hTF, the interaction between hTF and TbpB stabilizes iron binding, where the formation of the holo-hTF-TbpB complex is proposed to block protonation of a critical residue (H349) in hTF which acts as a pH-dependent trigger for iron release (187, 188); in this manner, iron is maintained within holo-hTF-TbpB until delivery to TbpA.

TbpA is similar to other TonB-dependent outer membrane receptors, as described previously. TbpA, however, is substantially larger (~20%) than other TBDTs, owing to extensive extracellular loop regions extending from both the β-barrel and plug domains, the latter of which has been proposed to function as a sensor for ligand binding (186). In contrast to TbpB, TbpA induces a large conformational change in hTF upon binding, where an α-helical extension of TbpA inserts itself into the iron-binding cleft of the hTF C-lobe, effectively prying the iron coordination site partially open and thus facilitating iron release. Transfer of ferric iron from TbpB to TbpA for subsequent uptake is mediated by the formation of a TbpB-holo-hTF-TbpA complex, which forms a bounded compartment that directs ferric iron toward the TbpA plug domain, although the mechanism by which this occurs has not yet been fully elucidated (186). Transport of insoluble ferric iron through TbpA is thought to be mediated by transient binding of iron to a conserved motif (EIEYE) in the plug domain (189).

As with TbpB, TbpA of N. meningitidis binds several human-specific residues on hTF (186), further explicating the specificity of these Tbps for hTF. The humanspecific tropism of N. meningitidis and N. gonorrhoeae is due in part to the specificity of TbpA/TbpB for hTF and

### TABLE 2: Examples of siderophore and transferrin-binding protein-dependent iron acquisition systems essential for virulence

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Siderophore</th>
<th>Model*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter baumannii</td>
<td>Acinetobactin</td>
<td>Mouse (i.p.)</td>
<td>283</td>
</tr>
<tr>
<td>Bacillus anthracis</td>
<td>Petrobactin</td>
<td>Mouse (s.c.)</td>
<td>231</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>Enterobactin</td>
<td>Chick</td>
<td>284</td>
</tr>
<tr>
<td>Dickeya damsii</td>
<td>Chrysobactin</td>
<td>African violet</td>
<td>285</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>Aerobactin</td>
<td>Mouse (s.c. and i.p.)</td>
<td>286</td>
</tr>
<tr>
<td></td>
<td>Yersiniabactin</td>
<td>Mouse (i.n.)</td>
<td>287</td>
</tr>
<tr>
<td>Legionella pneumophila</td>
<td>Legiobactin</td>
<td>Mouse (i.t.)</td>
<td>288</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>Mycobactin</td>
<td>Mouse (aerosol)</td>
<td>289</td>
</tr>
<tr>
<td>Pantoea stewartii</td>
<td>Aerobactin</td>
<td>Sweet Corn</td>
<td>290</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>Yersiniabactin</td>
<td>Mouse (t.u.)</td>
<td>291</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Pyochelin</td>
<td>Mouse (i.n. and i.m.)</td>
<td>292</td>
</tr>
<tr>
<td></td>
<td>Pyoverdine</td>
<td>Mouse (i.n. and i.m.)</td>
<td>292</td>
</tr>
<tr>
<td>Pseudomonas syringae</td>
<td>Pyoverdine</td>
<td>Tobacco plant</td>
<td>293</td>
</tr>
<tr>
<td>Salmonella enterica</td>
<td>Enterobactin</td>
<td>Mouse (i.p.)</td>
<td>294</td>
</tr>
<tr>
<td></td>
<td>Salmochelin</td>
<td>Mouse (o.g.)</td>
<td>219</td>
</tr>
<tr>
<td>Shigella flexneri</td>
<td>Aerobactin</td>
<td>Chicken embryo</td>
<td>295</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Staphyloferrin A</td>
<td>Mouse (s.c.)</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>Staphyloferrin B</td>
<td>Mouse (i.v.)</td>
<td>213</td>
</tr>
<tr>
<td>Yersinia pestis</td>
<td>Yersiniabactin</td>
<td>Mouse (i.v.)</td>
<td>296</td>
</tr>
<tr>
<td>Actinobacillus pleuropneumoniae</td>
<td>Tbp</td>
<td>Pigs (aerosol)</td>
<td>297</td>
</tr>
<tr>
<td>Neisseria meningitidis</td>
<td>Tbp</td>
<td>Mouse (i.p.)</td>
<td>298</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae</td>
<td>Tbp</td>
<td>Human male (urethritis)</td>
<td>299</td>
</tr>
</tbody>
</table>

* i.n., intranasal; i.m., intramuscular; i.p., intraperitoneal; i.t., intratracheal; i.v., intravenous; o.g., orogastric; s.c., subcutaneous.

### TABLE 3: Examples of inorganic iron uptake systems essential for virulence

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Uptake system</th>
<th>Model*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campylobacter jejuni</td>
<td>Feo</td>
<td>Piglet</td>
<td>300</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Feo</td>
<td>Mouse (o.g.)</td>
<td>301</td>
</tr>
<tr>
<td>Francisella tularensis</td>
<td>Feo</td>
<td>Mouse (i.d.)</td>
<td>302</td>
</tr>
<tr>
<td>Helicobacter pylori</td>
<td>Feo</td>
<td>Mouse (oral)</td>
<td>221</td>
</tr>
<tr>
<td>Legionella pneumophila</td>
<td>Feo</td>
<td>Mouse (i.t.)</td>
<td>303</td>
</tr>
<tr>
<td>Porphyromonas gingivalis</td>
<td>Feo</td>
<td>Mouse (s.c.)</td>
<td>304</td>
</tr>
<tr>
<td>Salmonella enterica</td>
<td>Sit</td>
<td>Mouse (i.p. and oral)</td>
<td>305</td>
</tr>
<tr>
<td>Xanthomonas oryzae</td>
<td>Feo</td>
<td>Rice plants</td>
<td>306</td>
</tr>
</tbody>
</table>

* i.d., intradermal; i.t., intratracheal; o.g., orogastric; s.c., subcutaneous.
has largely precluded the use of animal models for meningococcal and gonococcal infections. The recent development of transgenic mice expressing hTf represents a major breakthrough for studying the pathogenicity of Neisseria spp. (190, 191). Interestingly, TbpA binds several distinct transferrin residues that have evolved rapidly within primate lineages, suggesting that co-evolution of pathogenic Neisseriaceae, Pasteurellaceae, and Moraxellaceae with their primate host has driven adaptive changes within transferrin to evade this particular aspect of bacterial iron piracy (192). In contrast, the binding site of TbpB on hTf partially overlaps that of the mammalian transferrin receptor, TfR (187), thus protecting the TbpB binding site from rapid evolution that would otherwise mitigate binding of TbpB to hTf.

In addition to transferrin use, many pathogenic Neisseriaceae and Moraxellaceae are capable of exploiting lactoferrin as an iron source (193). Comprised of a TonB-dependent outer membrane lactoferrin-binding protein (LbpA) (194) and an accessory lactoferrin-binding lipoprotein (LbpB) (195), the lactoferrin acquisition system functions analogously to the TbpA/TbpB system described above, with notable differences. LbpB purportedly binds the N-lobe of holo-lactoferrin, in contrast to the binding of TbpB to the C-lobe of holo-transferrin (196). Further, LbpB appears to stabilize the iron-loaded form of lactoferrin not through manipulating a pH-dependent switch, but rather, by barring conformational changes that allow for iron release from holo-lactoferrin (196). Last, LbpB fulfills the unique function of conferring protection against the antimicrobial cationic peptide, lactoferricin, a degradation product of lactoferrin (197), by sequestering the peptide at negatively charged residues predominantly within the C-lobe of the lipoprotein and by potentially sheltering bound lactoferrin from proteolysis (196, 198, 199).

Despite these differences, it is thought that key structural and functional features of TbpA/TbpB and LbpA/LbpB are maintained. Notably, the α-helical extension of TbpA that was proposed to help extract iron from holo-transferrin is structurally conserved in LbpA, and thus the mechanism of iron release between these two proteins is believed to be comparable (196). Further, both TbpA and LbpA possess a periplasmic loop region that may mediate docking of the ferric-iron-binding protein A (FbpA) (200). Interestingly, FbpA, which is structurally similar to a single lobe of eukaryotic transferrin, effectively serves as a bacterial transferrin, shuttling insoluble and potentially toxic Fe³⁺ and small Fe³⁺-complexes between biological membranes (201). FbpA delivers Fe³⁺ to its associated permease, FbpBC, which together comprise an ABC transporter that represents a “nodal point” for ferric iron utilization in a number of Gram-negative pathogens (201–204). Indeed, FbpABC is required for iron uptake from transferrin, lactoferrin, and ferric iron chelates, such as ferric citrate and iron pyrophosphate in pathogenic Neisseria spp. (203–205). Together, TbpA/TbpB and LbpA/LbpB, coupled with FbpABC, form a distinct mechanism by which mucosal pathogens lacking endogenous siderophore production can exploit predominant iron sources within their host environment.

Siderophores

In contrast to the relatively limited prevalence of transferrin- and lactoferrin-binding proteins among bacterial pathogens, siderophores are ubiquitously employed as a tool to pirate iron from host glycoproteins and to scavenge residual free iron from the external milieu. Synthesized and secreted in response to iron deprivation,
siderophores are low molecular weight (generally less than 1 kDa), soluble molecules with high specificity and affinity for Fe$^{3+}$ (206). Enterobactin, a siderophore produced by Gram-negative enterics, is one of the strongest iron chelators known, with an estimated affinity of $10^{-52}$ M (207). Not only are siderophores widely expressed by both pathogenic and nonpathogenic bacteria, but they are often produced by fungi and graminaceous plants to promote growth under iron-limited conditions (208), and 2,5-DHBA was most recently identified as a mammalian siderophore (2, 3). To date, several hundred siderophores have been identified, over 270 of which have been structurally characterized. This has revealed a diverse array of structures yet a limited number of iron-coordinating functional groups (for a comprehensive list see Appendix 1 in reference 208). Broadly, siderophores can be categorized based on the functional groups involved in iron coordination and include catecholate, hydroxamate, and α-hydroxycarboxylate types. A fourth, nondescript category is comprised of siderophores employing more than one of the aforementioned classes of coordinating moieties and is thus referred to as “mixed type” (206). Regardless of type, siderophores tend to stably coordinate a single Fe$^{3+}$ ion in a hexadentate fashion, with oxygen atoms serving as the most common ligand (208).

The biosynthesis of siderophores typically follows one of two modes of assembly, delineated by the requirement, or lack thereof, for large multimodular enzymatic scaffolds referred to as nonribosomal peptide synthetases (NRPSs). NRPS-based siderophore synthesis involves the activation and incorporation of nonproteinogenic amino acids into an elongating chain, the sequence of which is dictated by the order of the NRPS domains, in lieu of an RNA template (for a detailed discussion of NRPS siderophore synthesis, see reference 209). In addition to the synthesis of numerous macrocyclic, or “aryl capped,” peptidic siderophores (206), including enterobactin, bacillibactin of Bacillus spp. (Fig. 7), mycobactin of M. tuberculosis, yersiniabactin of Yersinia spp., and vibriobactin of V. cholerae, NRPS enzymes also give rise to antimicrobial peptides and

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**FIGURE 7** Structures of representative catechol-containing stealth and nonstealth siderophores. Stealth siderophores are not bound by mammalian siderocalin.

<table>
<thead>
<tr>
<th></th>
<th>Enterics</th>
<th>Bacillus spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-stealth</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobactin</td>
<td><img src="image" alt="Enterobactin" /></td>
<td><img src="image" alt="Bacillibactin" /></td>
</tr>
<tr>
<td><strong>Stealth</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salmochelin S4</td>
<td><img src="image" alt="Salmochelin S4" /></td>
<td><img src="image" alt="Petrobactin" /></td>
</tr>
</tbody>
</table>
peptide-based antibiotics such as penicillin, daptomycin, and vancomycin (209). In contrast, NRPS-independent siderophore (NIS) synthesis employs an alternative family of synthetases, which catalyze the condensation of alternating amino alcohols, alcohols, dicarboxylic acids, and diamines via amine or ester bond formation, giving rise to nonpeptidic siderophores (for a detailed discussion of NIS siderophore synthesis, see reference 210). The majority of carboxylate and α-hydroxycarboxylate type siderophores are synthesized using NIS-type pathways, including staphyloferrins A and B of S. aureus, alcaligin of Bordetella pertussis and Bordetella bronchiseptica, and aerobactin of the Enterobacteriaceae (208). Interestingly, petrobactin (Fig. 7), which is produced by pathogenic Bacillus spp., is synthesized using a unique NIS/NRPS hybrid system (211).

To mediate iron uptake from the external milieu, newly synthesized siderophores must first be exported from the cytoplasm. Siderophore export is, to date, poorly characterized, but in both Gram-negative and Gram-positive bacteria, transport across the cytoplasmic membrane appears to be mediated by exporters of the major facilitator superfamily (212). Subsequent transport across the outer membrane in Gram-negative bacteria is performed by multidomain efflux pumps of the resistance-nodulation-cell division or ABC transporter-type families, and most often these export proteins are encoded from the biosynthetic locus of the associated siderophore. As discussed above, the transport of iron-loaded siderophores back across the Gram-negative outer membrane is mediated by specific TBDTs, and in both Gram-negative and Gram-positive pathogens, ABC transporters facilitate passage of the siderophore, intact, across the cytoplasmic membrane (Fig. 2). While some SBPs and ABC transporters are highly specific for a given siderophore, others possess broad substrate specificity for catecholate, carboxylate, or α-hydroxycarboxylate-type siderophores and, as such, may allow for the appropriation of siderophores from heterologous microorganisms.

Bacteria capable of exploiting these “xenosiderophores” may be afforded a competitive advantage in polymicrobial communities, where they are potentially able to pilfer multiple iron-chelating molecules without the energetic demands of synthesizing siderophores de novo. Indeed, some bacteria including S. lugdunensis (157), L. monocytogenes (12), and S. pyogenes, are wholly dependent on the uptake of xenosiderophores to access the transferrin-iron pool within the host, and the general trend is for the expression of several more siderophore importers than exporters overall.

The conserved ferric hydroxamate uptake (Fhu) system is but one example of a broad substrate range Fe³⁺-siderophore importer, homologs of which are found in each of the aforementioned organisms, along with many other Gram-negative and Gram-positive pathogens. Staphylococcus spp. and Bacillus spp. are additionally capable of utilizing catecholate-type siderophores, as well as ferrated catecholamine stress hormones, such as epinephrine, norepinephrine, and/or dopamine as “pseudosiderophores” using the staphylococcal siderophore transporter, SstABCD (213), and FeuABCD (214), respectively. Notably, catecholamine stress hormones do not directly strip iron from transferrin but, rather, promote its reduction from Fe³⁺ to Fe²⁺, thus facilitating iron release from transferrin and allowing for uptake by the hormone (215).

Upon delivery of the iron-loaded siderophore to the cytoplasm, two possible mechanisms exist for the release of iron for cellular processes: (i) reductive iron release from the siderophore using a ferrisiderophore reductase, leaving the molecule intact for possible recycling, and (ii) reduction of iron concurrent with hydrolysis of the siderophore backbone, as has been shown for tris-catecholate siderophores such as enterobactin and bacillibactin (206). The dissociation of iron from the intact siderophore is considered to be the more prevalent method, because specialized enzymes are not required and reutilization of the siderophore would ostensibly be more energetically favorable.

Stealth siderophores

In defense against siderophore-mediated iron piracy, neutrophils of the mammalian host secrete NGAL, an acute-phase protein capable of sequestering bacterial siderophores; unsurprisingly, bacterial invaders have responded by evolving tactics to counter this affront to their acquisition of iron. NGAL coordinates ferrisiderophores within a cup-shaped ligand-binding pocket (calyx) that is large, broad, and shallow and is uniquely lined with polar and positively charged residues (216, 217). As such, the NGAL calyx is ideally suited to the sequestration of negatively charged ferric siderophores, such as the catecholates. To circumvent the effects of NGAL, some pathogenic bacteria elaborate more than one siderophore, often including an NGAL-sensitive (e.g., catecholate) and an NGAL-immune siderophore (e.g., a structurally modified aromatic or one that simply lacks aromatic-binding motifs). Siderophores that evade the effects of NGAL are aptly referred to as “stealth siderophores” and are often associated with virulence potential. The production of a stealth siderophore is
exemplified by uropathogenic *E. coli* and *Salmonella enterica* serovars, where iron acquisition by NGAL-sensitive enterobactin is complemented by production of glucosylated enterobactin derivatives, such as salmochelin S4 (Fig. 7) (218); the addition of bulky glucose residues to the catechol moieties of the siderophore renders it sterically discordant for NGAL-binding. Indeed, despite the extremely high affinity of enterobactin for Fe\(^{3+}\), salmochelin appears to be required for the virulence of *S. enterica* (219). Similarly, pathogenic *Bacillus* spp. secrete both bacillibactin and petrobactin, the latter of which bears 3,4-DHBA moieties in lieu of the common 2,3-DHBA moieties, where the position of the carboxylates also renders the siderophore incompatible for binding by NGAL. Petrobactin is uniquely required for the virulence of *B. anthracis* (211).

**Extracellular Ferric Iron Reductases**

In theory, bacterial pathogens could utilize cell surface–associated or secreted ferric iron reductases, which reduce Fe\(^{3+}\) to Fe\(^{2+}\), not only to access insoluble Fe\(^{3+}\)-precipitates, but also to nonspecifically liberate iron from host iron complexes such as transferrin, lactoferrin, and ferritin (Fig. 1). The uptake of solubilized Fe\(^{2+}\) through the cell envelope occurs by way of inorganic iron permeases (Fig. 2), obviating the need for dedicated uptake mechanisms for each individual iron chelate. Indeed, extracellular and/or cell surface–associated reductase activity has been reported for *H. pylori* (220, 221) and the facultatively intracellular, ruminant pathogen *Mycobacterium avium* subsp. *paratuberculosis* (222), although the molecular determinants and substrate specificities of these enzymes are not well defined. Similarly, *L. monocytogenes* is capable of mobilizing iron from a wide range of complexes, including transferrin, lactoferrin, hemoglobin, catecholamine stress hormones, and exogenous siderophores, through what is thought to be a potent broad-substrate-specificity ferric iron reductase (223–226). A ferric reductase (FepB) recently described in *L. monocytogenes* functions as part of the ferric iron permease system, FepABC (for Fe\(^{2+}\)-dependent peroxidase), but it is unclear if the aforementioned activity can fully be ascribed to this enzyme (227). Interestingly, homogentic acid of *Legionella pneumophila* and the associated pyomelanin pigment, HGA-melanin, were both shown reduce Fe\(^{3+}\) complexes such as transferrin and ferritin, where mobilized Fe\(^{2+}\) was subsequently transported by the ferrous iron permease, FeoB (228, 229). Notably, siderophore production by *L. pneumophila* was found to be inversely correlated to homogentic acid–melanin secretion, suggesting that the two mechanisms are likely differentially regulated and may fulfill distinct roles in iron acquisition by this bacterium (228).

The interplay between well-defined iron acquisition strategies and ferric iron reductases has not yet been investigated, nor is it known if these enzymes contribute to the *in vivo* survival or pathogenicity of these organisms. It is interesting to speculate that ferric iron reductases may provide a means of accessing host intracellular iron stores, such as ferritin, but this notion has only been preliminarily investigated in *M. avium* subsp. *paratuberculosis*, where ferric reductase expression was observed in an association with intracellular mycobacteria of naturally infected bovine intestinal tissue (222). Clearly, while reductive iron release seems to be a viable means for pathogens to access a multitude of host iron complexes, to which they may not have specific receptors or acquisition pathways, it is a currently underappreciated and underexplored facet of bacterial iron acquisition.

**INORGANIC IRON TRANSPORT**

While Fe\(^{2+}\) uptake is important, particularly in anoxic and/or acidic environments, such systems are sparsely characterized in most microorganisms, owing to the obscuring influence of siderophore and heme-iron uptake systems. In Gram-negative bacteria, it is believed that ferrous iron ions are capable of diffusing across the outer membrane in a TonB-independent fashion and that elemental iron is actively transported across the cytoplasmic membrane. Two inorganic iron acquisition systems are widely conserved in both Gram-positive and Gram-negative pathogens: the ferrous iron transport system, Feo (see Table 3), and functional analogs of Fet3p-Ftbp1, a high-affinity, reductive iron permease in *Saccharomyces cerevisiae* (230).

**FeoAB(C): a Dedicated Ferrous Iron Transporter**

The *Enterobacteriaceae*, including key human pathogens such as pathogenic *E. coli*, *Shigella* spp., *Salmonella* spp., and *Y. pestis*, acquire inorganic iron in part through the highly conserved and dedicated ferrous iron transporter, Feo. In most γ-proteobacteria, Feo is encoded from the three-gene operon *feoABC* (231) and is regulated by Fur and anaerobic transcriptional activators, Fnr and/or AcrA, in response to iron and oxygen deprivation, respectively (232, 233). Surprisingly, despite the initial identification of Feo in *E. coli* K-12
nearly 30 years ago (234), the functions of the individual components of transporters are still somewhat unclear. FeoB is an integral membrane protein and likely functions as the main Fe$^{2+}$-permease, although it lacks homology to other known Fe$^{2+}$-transporters (231, 233). The activity of FeoB requires binding of GTP to a G protein domain located within its N-terminal cytoplasmic region, and while mutation of this binding site abolishes Fe$^{2+}$ transport (236), it is not known whether GTP hydrolysis energizes iron uptake by FeoB. FeoA is a small, hydrophilic, cytoplasmic protein of unknown function, bearing homology to eukaryotic Src homology-3 domains, which often facilitate protein-protein interactions. While conflicting evidence exists concerning whether FeoA interacts directly with FeoB, it is nevertheless required for FeoB-mediated Fe$^{2+}$ acquisition in S. enterica, V. cholerae, and E. coli (232, 237, 238).

FeoC is another small, hydrophilic, cytoplasmic protein that is only readily apparent in the γ-proteobacteria, although genes encoding other small proteins of undefined function are present in proximity to feo in other organisms (235). FeoC contains an Fe-S cluster and a putative winged-helix DNA-binding motif (232, 234, 239) and, accordingly, was once thought to serve as a transcriptional regulator of feoABC (235). Instead, it appears, at least in the case of S. enterica, that FeoC binds and protects FeoB from proteolytic degradation in iron-deplete, anoxic environments (240). FeoC itself is subject to oxygen-sensitive proteolysis, where the Fe-S cluster is thought to serve as an oxygen sensor, promoting degradation of the protein during aerobicosis (241). Thus, FeoC may provide assurance that FeoB is stably expressed when oxygen and iron are limiting and that it is rapidly degraded when they are not, thus limiting further control over Fe$^{2+}$ acquisition by the bacterium. Together, FeoABC represents a key means by which enteric pathogens, in particular, acquire iron in microaerophilic or anaerobic niches of the gastrointestinal tract. Indeed, feoABC has been implicated in the virulence of many of these pathogens, as summarized in Table 3. While considerable gaps exist in our understanding of how FeoABC functions in Fe$^{2+}$ uptake in the γ-proteobacteria, even less is known about the transporter in Gram-positive pathogens. Homologs of feoAB have been identified in a number of Gram-positive pathogens, including B. anthracis, S. aureus, and L. monocytogenes, and although the operon is upregulated during iron-restricted growth in the latter two (235, 242, 243), Feo has yet to be functionally characterized in any of these organisms.

EfeUOB/FepABC: a Conserved but Enigmatic Pathway for Inorganic Iron Uptake

The Fur-regulated, acid-induced elemental ferrous iron uptake system of E. coli, EfeUOB, is a tripartite inorganic iron transporter that bears homology to the Fe$^{2+}$ acquisition system Fet3p-Ftrp1 of baker’s yeast (244, 245). Homologs of efeUOB are widely distributed among many bacterial phyla, although for nearly every characterized system, the preferred substrate remains a matter of debate. In E. coli, EfeUOB has been implicated not only in the acquisition of Fe$^{2+}$ and Fe$^{3+}$, but also in the extraction and subsequent uptake of iron from heme (246), as has similarly been postulated for the staphylococcal EfeUOB ortholog, FepABC (247, 248). In B. subtilis, EfeUOB is involved in the acquisition of both Fe$^{2+}$ and Fe$^{3+}$ (249), whereas a similar four-component system in pathogenic Bordetella spp. and Brucella spp. appears to be a dedicated ferrous iron transporter (FtrABC) (250, 251). As discussed above, FepABC of L. monocytogenes may facilitate access to a plethora of host Fe$^{3+}$-complexes through robust ferric reductase activity, but a substrate of this transporter has not yet been defined (227).

EfeU and FepC are integral transmembrane proteins bearing homology to the yeast ferric iron permease, Ftrp1. While efeUOB is required for Fe$^{2+}$ acquisition in E. coli under acidic conditions (244, 245), it is likely that the EfeU (FepC) serves as a transporter of Fe$^{3+}$, where Fe$^{2+}$ captured from the external milieu is oxidized to Fe$^{3+}$ by the uncharacterized substrate-binding protein, EfeO (FepA), prior to uptake. In most organisms, EfeO (FepA) possesses a putative divalent metal cation-binding motif (GxHxxE) and an N-terminal cupredoxin (Cup) domain, the latter of which is thought to confer the aforementioned ferroxidase activity, similar to the yeast multicopper oxidase, Fet3p (252). Contention regarding the substrate specificity of EfeUOB/FepABC largely hinges on the function of EfeB (FepB), an extracytoplasmic protein of the dye-decolorizing heme peroxidase (DyP) superfamily. Members of the DyP family possess highly varied functions, and in E. coli and S. aureus, EfeB (and a paralog YfeX) and FepB, respectively, have been credited with a novel heme deferrochelatase activity. Deferrochelation is a process wherein iron is removed from heme through a reductive process that, remarkably, conserves the porphyrin ring, thus yielding free iron and PPIX (247, 253, 254). Given that PPIX is not readily metabolized by bacteria and is potentially toxic, questions have been raised about the biological utility of generating PPIX as a byproduct of heme-iron extraction. As such, other research suggests
that EfeB, YfeX, and FepB may instead function as typical DyP peroxidases to oxidize porphyrinogens to porphyrins (255). In *B. subtilis*, EfeB does appear to function as a DyP peroxidase but uniquely functions as a switch between Fe$^{2+}$ and Fe$^{3+}$ acquisition in this organism. Notably, EfeO in *B. subtilis* lacks a Cup domain (and thus ferroxidase activity) and instead is capable of receiving Fe$^{3+}$ directly from the external milieu in the absence of EfeB. Under microaerobic conditions, when Fe$^{2+}$ predominates, EfeB oxidizes Fe$^{2+}$ to Fe$^{3+}$ prior to transfer to EfeO and subsequent uptake into the cytosol by EfeU (249). Although inconsistencies remain, it appears that EfeUOB/FepABC has distinctively evolved in specific microorganisms to facilitate access to the iron sources most frequently encountered and/or to which no other means of uptake exist. While investigations into the in *vivo* role of EfeUOB/FepABC are limited, orthologous transporters in both *S. aureus* (*fepABC*) and *Brucella abortus* (*ftrABCD*) have been implicated in the survival and virulence of these pathogens within murine models of infection (248, 251). Thus, inorganic iron acquisition via *fepUOB*/fepABC and similar ferric iron permeases should not be disregarded as an inconsequential means of iron uptake.

**IRON ACQUISITION SYSTEMS AS THERAPEUTIC TARGETS**

Given the essentiality of iron to bacterial pathogens and the known expression of iron acquisition systems within the host, these systems have evoked much interest in developing antibacterial strategies, for either the development of vaccines, the design of iron acquisition inhibitors, or the utility of iron acquisition systems to deliver toxic substances into the cell in a “Trojan horse” type of approach. Below, we present a very brief summary of a few examples of how iron acquisition systems are being used as therapeutic targets.

**Iron-Acquisition Proteins as Vaccine Targets**

For many pathogenic species of bacteria, there is no approved vaccine. Many proteins involved in iron acquisition satisfy several key requirements for vaccine candidates in that they are exposed at the cell surface, expressed *in vivo*, frequently essential to virulence, and are conserved within species. Space limitations dictate that we limit our discussion here to only two examples.

Several components of the Isd system in *S. aureus* have generated interest in vaccine development. IsdB, IsdA, and IsdH are immunogenic in laboratory animals and can provide a protective response in mice (256–259), and IsdB also elicited a strong immune response in rhesus macaques (260). The IsdB vaccine, V710, showed immunogenic properties in humans in phase I trials (261) and moved to phase II/III trials in adults, at which point, unfortunately, the trials on V710 were halted due to an inability of the vaccine to protect against postoperative *S. aureus* infection (262). Research continues to examine the utility of other Isd components as part of an effective *S. aureus* vaccine, and multivalent approaches appear to provide much promise. In one example, immunization of mice with a combination of IsdA and IsdB, along with two other surface-expressed proteins, SdrD and SdrE, substantially increased survival of *S. aureus*–challenged mice compared to unimmunized controls (259).

TbpA and TbpB have been examined as vaccine candidates for over two decades for *N. meningitidis* and *N. gonorrhoeae* (263). Denatured TbpA and/or TbpB from *N. meningitidis* elicited an immune response in animals and provided protection from *N. meningitidis* infection (264, 265), and humans generate an antibody response to these proteins (263, 266, 267). In contrast, *N. gonorrhoeae* TbpA and TbpB do not evoke a strong immune response in humans, because IgG, IgA, and IgM antibody levels in the serum of individuals with a previous gonococcal infection were low and comparable to the control group (268). However, a TbpB-cholera toxin B (Ctb) conjugate administered to mice had significantly higher antibody levels in serum than TbpB alone (269). Although there is currently a vaccine for *N. meningitidis*, it is not effective against serogroup B or *N. gonorrhoeae*. Because TbpB is conserved in all isolates of *N. meningitidis* and *N. gonorrhoeae*, it remains a promising vaccine candidate.

**Exploiting Iron Acquisition Systems for Antibiotic/Inhibitor Design**

Among the diverse array of siderophore structures are those that possess antibiotic properties, termed sideromycins. Naturally occurring sideromycins include albolomycin and the salmycins that contain the antibiotic moieties thioribosyl pyrimidine or aminoglycosides, respectively. The molecules gain access to the cell via the normal route of entry for the siderophore component. Sideromycins have not seen extensive use as therapeutics because of the development of rapid resistance, owing to mutations in the siderophore transport system or to the protein required for intracellular antibiotic activation. However, significant research has been performed to generate synthetic siderophore-antibiotic conjugates,
exploiting the versatility of synthetic iron chelators to improve efficacy as therapeutic agents. The reader is referred to a comprehensive review on the topic (270).

For bacteria for which siderophore synthesis is an important virulence determinant, siderophore biosynthesis inhibitors offer promise as a novel class of antibacterial. The biosynthetic pathway for catechol-type siderophores, such as mycobactin, yersiniabactin, enterobactin, and pyochelin, for example, proceeds from chorismate utilizing chorismate-utilizing enzymes such as salicylate synthase and isochorismate synthase. That these enzymes are not present in mammalian cells makes them ideal targets for bacterial inhibition. For a more detailed discussion on the development of inhibitors to chorismate-utilizing enzymes, we refer the reader to a recent review (271).

An alternative strategy to target iron metabolism in bacteria is through intoxication by alternative transition metals. Noniron metalloporphyrins, such as gallium-PPIX, have potent antibacterial activity against both Gram-negative and Gram-positive bacteria (272). These compounds exploit heme uptake systems as a means to enter cells, and since heme acquisition is important to many pathogens, these compounds offer exciting possibilities to treat problematic human pathogens. Alternatively, conjugating gallium to siderophores, such as deferoxamine mesylate, has been shown to be effective in killing P. aeruginosa, blocking biofilm formation, and decreasing corneal infection in rabbits when combined with gentamicin, as opposed to gentamicin treatment alone (273). These data provide the incentive to initiate research into the testing of this and other siderophore gallium conjugates for efficacy in other bacterial pathogens.

CONCLUSIONS

The past decade of study on iron acquisition strategies employed by human, animal, and plant pathogens has brought rapid progress and led to breakthroughs in our understanding of how pathogens have evolved to deal with iron limitation imposed by the host. Although this field has been one of the most intensely studied for decades, new and fascinating information continues to be uncovered with regard to the role of iron on bacterial physiology, metabolism, adaptation, and virulence. Important new insights into mechanisms of iron scavenging continue to be derived from a continuum of work from many laboratories in the disciplines of microbiology, cellular microbiology, pathogenesis, biochemistry, and structural biology. We are now closer than ever to seeing the translation of knowledge on iron acquisition systems into valuable inhibitors, antibiotics, and vaccines aimed at decreasing the burden of infectious disease by ever-evolving drug-resistant bacterial pathogens.

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