Published in final edited form as:

Chembiochem. 2020 July 16; 21(14): 1955–1967. doi:10.1002/cbic.201900778.

Iron Acquisition by Bacterial Pathogens: Beyond *tris*-Catecholate Complexes

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Abstract

The sequestration of the essential nutrient iron (Fe) from bacterial invaders that colonize the vertebrate host is a central feature of nutritional immunity and the “fight over transition metals” at the host-pathogen interface. The Fe quota for many bacterial pathogens is large, as Fe enzymes often comprise a significant share of the metalloproteome. Fe enzymes play critical roles in respiration, energy metabolism and other cellular processes by catalyzing a wide range of oxidation-reduction, electron transfer, and oxygen activation reactions. In this Concepts report, we discuss recent insights into a diversity of ways that bacterial pathogens acquire this essential nutrient beyond well-characterized *tris*-catecholate Fe^{III} complexes, in competition and cooperation with significant host efforts to cripple these processes. We also discuss pathogen strategies to adapt their metabolism to less-than-optimal Fe, and briefly speculate on what may be an integrated adaptive response to concurrent limitation of both Fe and Zn in the infected host.

Graphical Abstract

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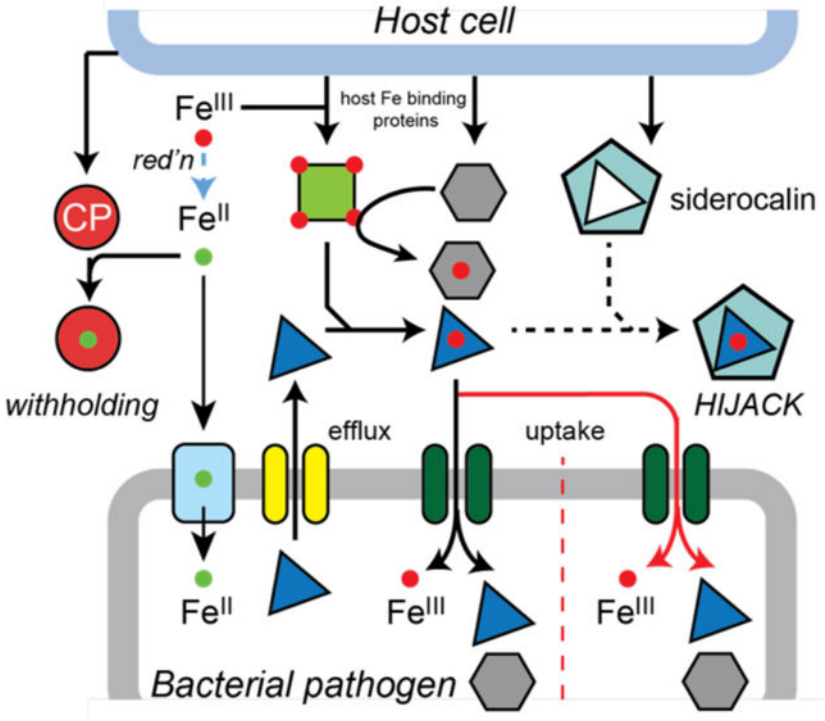
Conflict of Interest

The authors declare no conflict of interest.



Bacterial pathogens employ myriad strategies to acquire the essential nutrient iron. Here we highlight newly emerging features of bacterial Fe acquisition beyond classical Fe^{III}-siderophores, including the use of coordinatively unsaturated Fe^{III}-catechol complexes and Fe^{II} uptake. The bacterial adaptive response to host efforts to restrict Fe availability is also discussed.

Keywords

Iron; iron uptake, host-pathogen interface; metal nutrient acquisition; siderophore; catechols

1 Introduction

1.1 Iron in bacterial cellular metabolism

Iron (Fe) is among the most Earth-abundant transition elements and is extensively used by nearly all living organisms to perform an impressive array of chemical transformations. Fe-dependent catalysis impacts many aspects of metabolism, including energy metabolism, respiration, lipid metabolism, amino acid and cofactor biogenesis and DNA metabolism.[1] Fe enzymes play important roles in electron transfer processes and as oxidoreductases, and both functions derive from the accessibility of ferrous (Fe^{II}) and ferric (Fe^{III}) oxidation states. Fe-dependent mono- and dioxygenases often cycle to higher oxidation states, *e.g.*, Fe^{IV}, which is a potent oxidant. Fe speciation in proteins involves ionic mononuclear Fe and multi-metal (Fe-Fe, or mixed metal, *e.g.*, Ni-Fe) coordination complexes, Fe-heme or iron-sulfur (Fe-S) clusters, the latter of which are inorganic complexes of Fe with sulfide (S^{2-}), as [2Fe-2S] or [4Fe-4S] cubane structures. In a typical Fe-centric, facultative anaerobic, Gram-negative bacterium, *e.g., Escherichia coli*, characterized by an outer membrane (OM) and an

inner cytoplasmic membrane (CM) that collectively encompass the periplasm, Fe-containing proteins are estimated to comprise upwards of 85% of the metalloproteome,[2] which itself accounts for ≈30% of the entire proteome.[3] This metal quota is significant, with the next largest contributor to the metalloproteome zinc (Zn) metalloenzymes, which comprise the majority of what remains (≈15%; or 5–6% of a typical bacterial proteome).[4]

1.2 Fe bioavailability and the microenvironmental niche

Since the footprint of Fe-dependent metabolic activity is large, Fe withholding from a bacterial pathogen that colonizes the vertebrate host is foundational to the concept of nutritional immunity (see below).[5] Fe sparing[6] has emerged in response as a broadly employed strategy that bacterial cells use to lower their cellular demand during extreme Fe restriction during infection.[7] In addition, the successful pathogen employs a myriad of strategies to secure Fe from the surrounding microenvironment The latter helps to avoid the collateral damage of weakly complexed “free” Fe^{II} in an aerobic environment, which becomes a potent autocatalytic producer of oxidative stress, as highly reactive hydroxyl radicals (OH•) formed by the Fenton reaction. Fe^{II} will predominate in the reducing environment of the cytoplasm, and is considered a weakly competitive transition metal, which generally binds to protein sites with only modest affinity, only greater than that of Mn^{II}.[8] Some bacteria have evolved Fe^{II} efflux transporters, likely as a means to mitigate the impact of Fe^{II} excess,[6a, 9] particularly under conditions of host-derived oxidative stress.[9–10] Maintaining bioavailable Fe concentrations and optimal Fe speciation that is compatible with cellular viability during infection is controlled by metalloregulatory proteins that sense either Fe^{II}, *e.g.*, Fur (ferric uptake regulator),[11] Fe-S loads,[12] or in some cases, heme-Fe.[13] Fur, for example, represses the expression of the Fur regulon when Fe is replete.[8] As Fe becomes increasingly scarce, Fe^{II} dissociates from Fur, and Fur, in turn, sequentially releases from individual DNA operator sites in “waves”, driving a graded transcriptional response to cellular Fe deprivation.[6a] Fe^{II} sensing in many organisms is intertwined with oxidative stress resistance[14] and Mn^{II} acquisition[15] since Mn^{II} can substitute for Fe^{II} in some non-heme Fe enzymes, thus protecting these enzymes against Fe-catalyzed oxidative damage.[16]

Since Fe is clearly a precious commodity, bacteria have evolved a remarkable array of mechanisms to scavenge this nutrient from the environment. In this Concepts paper, we review recent progress in our understanding of Fe uptake by bacterial pathogens that goes beyond the well-studied Fe^{III}-*tris*-catecholate systems, exemplified by the Fe^{III}-enterobactin (enterochelin) extensively characterized in *E. coli* (Figure 1).[17] The work reviewed here is not meant to be comprehensive, but instead highlights newly emerging features of Fe acquisition and the bacterial adaptive response to host efforts to restrict the availability of this essential metal.

2 The tris-catecholate complexes

2.1 General features and regulation

Siderophores are small molecule, high affinity Fe^{III} chelators that harbor up to six coordinating donor atoms and typically form coordinatively saturated complexes with the

metal.[18] Four structural classes of siderophores have thus far been identified, and these include the catecholate, hydroxamate, carboxylate and mixed-type siderophores distinguished by their distinct combinations of Fe^{III} coordinating donors (Figure 2A).[19] The archetypal siderophore is enterobactin (Ent), a *tris*-catechol derivative of a cyclic triserine lactone. The metal coordinating motif is an *ortho* 2,3-dihydroxybenzoate (DHB) substituent coupled to the α-amino group of *L*-serine via an amide linkage. The cyclic trilactone core contains three ester linkages with three dihydroxybenzylserine (DHBS) metal coordinating groups that completely surround the Fe^{III} atom (Figure 2A). Fe^{III} binds with a stability constant of log K^{Fe} of ≈36 at pH 7.0 with a significant contribution of the chelate effect, and thus competes well with host mononuclear Fe^{III} complexes of the major serum Fe transport protein, *e.g.*, transferrin (log K^{Fe} of 22[20]) or lactoferrin. These ester linkages are susceptible to slow hydrolysis in aqueous solution [21] or in a reaction catalyzed by esterases (Figure 2A, *top*).

A typical Gram-negative bacterium like *E. coli* or *Salmonella enterica* sevovar Typhimurium harbors the biosynthetic machinery to synthesize Ent. The biosynthesis is typically controlled by Fur, which in *E. coli* regulates the expression of three Ent-related gene clusters, *fepDGC-entS*, *fepB-entC*, and *fepA-fes*.[22] The *fepDGC* genes encode an inner (cytoplasmic) membrane localized ATP-binding cassette (ABC) transporter to which FepB, the periplasmic solute binding protein (SBP), binds when bound to cargo Fe^{III}-Ent, and is transported across this membrane using the energy of ATP hydrolysis (Figure 3A). *entS* encodes the apo-Ent effluxer that pumps Ent into the periplasm and ultimately into the extracellular milieu where it captures Fe^{III}. Fe^{III}-Ent then binds to an outer membrane (OM)-integrated 22-stranded β-barrel receptor (FepA), which via a TonB-ExbB-ExbD-dependent transport process, is brought into the periplasm (Figure 3A).[23] Once across the cytoplasmic membrane via FepBCDG, Fe^{III}-Ent is subjected to esterase-catalyzed cleavage by Fes and/or reduction to Fe^{II} in the cytoplasm by resident ferric siderophore reductase(s); this makes Fe^{II} bioavailable to client biomolecules.[24] Other bacteria, including *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, lack the machinery to biosynthesize Ent, but express an OM receptor that specifically brings Fe^{III}-Ent into the periplasm (*P. aeruginosa* PfeA; Figure 3B).[25] Thus, Ent is a xenosiderophore for these organisms (Figure 1). This strategy of iron piracy is ubiquitous in polymicrobial niches, and highlights the importance to the bacterium of tapping any and all bioavailable sources of Fe in that niche.

2.2 Siderophore-dependent Fe^{III} acquisition across membranes

The ligand specificity of the Fe^{III}-siderophore uptake appears to lie largely at the level of the OM receptor in Gram-negative organisms (Figure 3A). Although the structure of the ligand-free OM Fe^{III}-Ent receptor *E. coli* FepA has been known for over twenty years,[23c] the structure of the FepA homolog from *P. aeruginosa*, PfeA, bound to Fe^{III}-Ent provides new insights into the transport mechanism.[23c] This structure reveals a series of large extracellular loops that capture Fe^{III}-Ent, coupled to an N-terminal globular plug domain that is inserted into the pore of the barrel (Figure 3B, C). Fe^{III}-Ent binds to these extracellular loops that extend above the barrel itself, which function as “fingers on a hand” to grab the ligand. Fe^{III}-Ent binding triggers a conformational change in the N-terminal plug domain that appears to push the Fe^{III}-Ent complex to a second, intramembrane binding site.

This allows the "Ton-box" on the plug domain to physically engage TonB which in turn triggers a relocation of plug domain to open a periplasmic gate to allow Fe^{III}-Ent uptake into the periplasm.[26] Although both FepA and PfeA transport Fe^{III}-Ent, PfeA also binds and transports other *tris*-catechol xenosiderophores, *e.g.*, protochelin, as well as a *bis*-catechol, Fe^{III}-azotochelin (Figure 2), which form complexes that are nearly isostructural with that of Fe^{III}-Ent [26a] and thus represents a further example of Fe piracy (Figure 1). Other OM receptors reported as catecholate-type siderophore uptake systems in *E. coli* are CirA and Fiu, the latter of which is a candidate receptor of Fe^{III}-$DHBS_n$ complexes (see Section 3).[27] The structure of Fiu reveals a selectively-gated cavity in the transporter that binds the Fiu substrate and appears to function in a *P. aeruginosa* PfeA-like two-step mechanism.[28]

In Gram-negative organisms, the Fe^{III}-siderophore complex enters the periplasm after crossing OM where it is captured by an SBP and then transported into the cytoplasm via an ABC-type active transporter (Figure 3A, *left*).[29] In Gram-positive organisms, SBPs are anchored to the cell membrane coupled with an ABC transporter and are termed lipoproteins (Figure 3A, *right*).[29] In these organisms, the lipoprotein SBP actively discriminates among various Fe^{III}-chelates, to form specific, high affinity, yet transport-competent complexes with their cargo.[30] For example, the major human pathogen *Staphylococcus aureus* synthesizes two citrate-derived polycarboxylate-type siderophores, staphyloferrin A (SA) and staphyloferrin B (SB) (Figure 2A)[31] The lipoprotein SBPs HtsA[32] and SirA[31a] bind with high affinity and specificity to only their cognate Fe^{III} complexes formed by SA and SB, respectively (Figure 3A). All metal-transporting SBPs and lipoproteins, including those that transport Fe^{III}-siderophore complexes, are members of the class III (cluster A) clade in which the two internally duplicated "lobes" are connected by a long α-helix or "brace" helix, with the cavity between the lobes defining the ligand binding pocket (see Figure 4B, below).[29] Ligand binding to class III SBPs results in little or no overall conformational change, unlike other SBP classes.[33] *E. coli* FepB, like FepA, has high specificity for Fe^{III}-Ent[34] and even a linear *tris*-catecholate Fe^{III}-siderophore such as agrobactin cannot be transported by FepB.[35]

Analogous transporters have been described for other siderophore classes in many human pathogens (Figure 2A). These include the *E. coli* ferrichrome (hydroxamate) uptake system, composed of the OM receptor FhuA and the Fe^{III}-ferrichrome binding protein FhuD.[36] In the Gram-negative pathogen, *A. baumannii*, a mixed type siderophore Fe^{III}-acinetobactin is first transported across the OM as 1:1 or 1:2 Fe^{III}-(pre)acinetobactin complexes through BauA,[37] which is then bound by the SBP, BauB, as a 1:2 Fe^{III}-acinetobactin complex (Figure 2).[38] There is evidence in some organisms that periplasm-resident esterases might process (hydrolyze) cyclic siderophores once brought across the OM, as shown for the *tris*-lactone esterase IroE which required for linear salmochelin uptake in uropathogenic *E. coli*.[24a] The extent to which this is generally true is not known, and competing models for IroE function exist, including hydrolysis of the metal-free *tris*-catecholate prior to export.[39]

2.3 The intracellular fate of Fe^{III}-siderophore complexes

Fe^{III} release once inside the cytoplasm is generally accomplished by two general strategies. These are covalent modification or hydrolysis of, for example, the *tris*-lactone siderophore,

and/or reduction of the bound Fe^{III} to Fe^{II}. Both lower metal-chelate stability, and in the case of the reduction to Fe^{II}, greatly enhances Fe aqueous solubility (Figure 3A).[40] In *E. coli* for example, the Fur-regulated cytoplasmic Fe^{III}-Ent esterase Fes hydrolyzes the *tris*-lactone ester bonds to generate DHBS monomers as the final product (Figure 2A, *top*).[39] Although each DHBS harbors the bidentate catechol motif, the complex is less thermodynamically stable than Fe^{III}-Ent. YqjH is a widespread NADPH, FAD-dependent Fe^{III} reductase in *E. coli* capable of promoting Fe^{II} release from a variety of *tris*-catecholates including Ent, vibriobactin and aerobactin;[41] others have been characterized in *S. aureus*.[40] The reduction efficiency for Fe^{III} bound to bidentate or simple organic ligands, such as DHBS and dicitrate, is high thus increasing the bioavailability of Fe^{II} in the cytoplasm.[41] These iron release mechanisms mentioned above are generally transcriptionally controlled by the global iron response regulator, Fur. YqjH is regulated by both Fur and YqjI, a transcriptional regulator that senses nickel toxicity in *E. coli*, as expected since nickel toxicity is known to disrupt Fe homeostasis.[42]

A cytoplasmic membrane associated [2Fe-2S] cluster protein, *E. coli* FhuF, is capable of reducing Fe^{III}-hydroxamate siderophore complexes, including those formed with ferrichrome and ferrioxamine B (Figure 2).[43] In addition, there is evidence to suggest that one of the *N*-hydroxyl groups of ferrichrome is subject to acetylation after Fe^{III} reduction so as not to negatively impact Fe speciation in the cytoplasm; the acetylated ferrichrome is then effluxed into the extracellular space where it is slowly hydrolyzed.[44] In this way, ferrichrome is recycled so as to maximize the benefit of energy-intensive *de novo* siderophore biosynthesis.

3. Coordinatively unsaturated bis- and mono-catecholate Fe^{III} complexes as Fe sources

3.1 General considerations

In order to form high affinity complexes with Fe^{III}, a typical siderophore forms coordinate-covalent bonds with all six donor atoms derived from a single molecule, like those described for the cyclic *tris*-catecholate Ent (Figure 2A,B). However, atypical Fe^{III} complexes can also be used by bacterial pathogens to acquire Fe. These can be operationally grouped into two groups: coordinatively *saturated* and coordinatively *unsaturated* Fe^{III} complexes (Figure 2B). Coordinatively saturated Fe^{III} complexes incorporate six donor atoms derived from one, two or three liganding molecules and thus might have mixed ligand donor sets (Figure 2B, *left*), an early example of which is the Fe^{III} complex formed by pyochelin (tetradentate) and cepabactin (a bidentate, monomeric hydroxamate ligand) in *Pseudomonas cepacia*.[45] Coordinatively unsaturated complexes, in contrast, are formed by one tetradentate dimer molecule or by two bidentate monomers with the remaining two coordination sites occupied by solvent (Figure 2B, *right*). Even though these coordinatively unsaturated Fe^{III} complexes are characterized by lower overall Fe^{III} stability constants, they still function as biologically important Fe^{III} scavengers. This structural diversity may offer significant growth advantages in specific tissue niches, particularly for those bacteria that do not synthesize their own siderophores. These bacteria include the obligate human pathogens *Neisseria meningitidis* and *Neisseria gonorrhoeae*,[46] the intestinal pathogen *Campylobacter jejuni*[47] and two

respiratory pathogens capable of colonizing the human nasopharynx, *Streptococcus pneumoniae* and *Haemophilus influenzae*.[48]

3.2 Coordinatively unsaturated tetra- and bidentate catecholate siderophores

The most well-studied coordinately unsaturated Fe^{III}-siderophores are the tetradentate and the *bis*-catecholate-type siderophores (Figure 2A). These siderophores include, but are not limited to, the endogenously synthesized *bis*-catecholates azotochelin and azotobactin d from *Azotobacter vinelandii*,[49] pyochelin from *Pseudomonas* ssp.[50] and amonabactin from *Aeromonas* spp.[51] The hydrolysis products of Ent including linear tri-DHBS, di-DHBS and DHBS monomers (Figure 2A) are also present, and although their origin within a complex milieu of Fe^{III}-siderophores at infection sites[52] is often not known, they allow bacteria to grow under iron-limited conditions.[27b, 53] In addition to these siderophore hydrolysis products of likely bacterial origin, other host-derived candidate Fe^{III} chelators are the monomeric catechols, *e.g.*, the catecholamines, human stress hormones that localize to sites of infection[54] at concentrations approaching 10 μM.[55] The catecholamine norepinephrine (NE) (Figure 2A) is known to enhance the proliferation of a number of bacterial pathogens, including *S. pneumoniae*, under Fe-limited growth conditions,[47b, 56] particularly in tissue niches where transferrin is the primary available iron source.[57] Although some organisms, including *Neisseria* spp. and *H. influenzae* can extract Fe^{III} from transferrin directly via an OM receptor,[46, 58] others cannot. It is thought that the auto-oxidation of NE reduces Fe^{III} to Fe^{II}, thus triggering iron release from transferrin;[59] the released Fe is then re-oxidized to Fe^{III} upon capture by NE. Consistent with this, it is known that catecholamines form 2:1 or 3:1 (Figure 2B) Fe^{III}-catecholamine complexes at physiological pH.[60] The generality of these findings and their relevance during infections is not yet known. To explore this further, the metabolic fate of these catecholates will have to be elucidated as described in the context of Fe^{III}-bacillibactin metabolism in *Bacillus subtilis*.[61]

3.3 Iron acquisition via coordinately unsaturated tetra- and bidentate Fe^{III} complexes

How do these simple, coordinately unsaturated Fe^{III} complexes get into cells? Recent studies confirm that catecholate siderophore Fe^{III} complexes cross the OM via a TonB dependent OM receptor CfrA in *Campylobacter* ssp.; as expected, inactivation of CfrA strongly impairs norepinephrine-dependent growth (Figure 4A).[47b, 56a, 56b, 62] A periplasmic esterase Cee in *C. jejuni*, capable of hydrolyzing Ent to DHBS monomers analogous to that carried out by the cytoplasmic Ent esterase Fes in *E. coli*, [63] becomes essential when Fe^{III}-Ent is the sole iron source in culture, consistent with the hypothesis that periplasmic hydrolysis of Ent is required for Fe^{III} uptake in this organism.[63] This finding predicts the presence of a periplasmic SBP that is specific for coordinately unsaturated Fe^{III}-NE and Fe^{III}-Ent hydrolysis products localized to the cytoplasmic membrane (see Figure 2B, *right*); this is, in fact, CeuE, from the ABC transporter, Ceu.[64] Although the structure of CeuE (Figure 4B) reveals a positively charged, ligand binding pocket reminiscent of the Fe^{III}-Ent binding pocket of *E. coli* FepB or *P. aeruginosa* PfeA,[56c] CeuE binds Fe^{III}-Ent relatively weakly, while coordinatively unsaturated Fe^{III}-di-DHBS or Fe^{III}-$[DHBS]_2$ complexes bind with far higher affinity.[56c] The crystal structures of CeuE bound to a series of 1:1 Fe^{III} complexes of an artificial tetradentate, *bis*-catecholate complex, 4-LICAM, establish why: the two open

Fe^{III} coordination sites on one side of the *bis*-catecholate chelate are occupied by a conserved tyrosine (Y288), buried in the binding pocket, and a conserved histidine (H227) derived from a flexible loop that covers the binding pocket (Figure 4C,D).[56c, 65]

This Fe^{III} acquisition system in *C. jejuni* establishes the physiological importance of coordinatively unsaturated Fe^{III}-siderophore complexes as nutritional iron sources. In fact, CeuE-type SBPs that conserve both Tyr and His ligands are found in Gram-negative and Gram-positive pathogens.[56c] This includes *Vibrio cholerae* VctP[66] which is known to bind the Fe^{III} complex of a tetradentate *bis*-catecholate, salmochelin S1, preferentially over hexadentate siderophores. A similar CueE-type SBP, SstD, is found in *Staphylococcus aureus* and has been shown to be required for Ent-dependent Fe^{III} acquisition and catecholamine-stimulated growth under severely iron-restricted conditions.[31b] This occurs despite the fact that both *V. cholerae* and *S. aureus* biosynthesize their own siderophores (Figure 2). The capacity to utilize hydrolysis products of *tris*-catecholate siderophores in polymicrobial niches is likely important, since intact siderophores may be limiting due to scavenging by other bacteria or sequestration by host siderocalin (Section 5).[56c, 66]

4. Fe^{II} uptake

4.1 Fe^{II} bioavailability at the host-pathogen interface

Although Fe^{III} has long been considered the nutritionally important oxidation state of Fe at the host-pathogen interface, Fe^{II} can also be bioavailable particularly in anaerobic and/or acidic niches, both of which would enhance the solubility of Fe^{II}.[67][68] However, Fe^{II} can be accessible in other microenvironments as well. For example, extracellular Fe^{II} bioavailability may be increased by redox active metabolites like phenazines (see Figure 1) that are secreted by pathogens like *P. aeruginosa* and are capable of reducing Fe^{III} to Fe^{II}.[68a, 69] Weakly complexed Fe^{II} can diffuse freely into the periplasm through outer membrane porins in Gram-negative organisms; alternatively, a ferric reductase anchored to the cytoplasmic membrane functions to reduce periplasmic Fe^{III} to Fe^{II},[70] which is then transported into the cytoplasm by the Feo system (Section 4.2). The source of electrons for Fe^{III} reduction by these ferrireductases could be heme,[70b] NADH[70a] or flavins.[71] In another variation on Fe^{II} uptake, the obligate intravacuolar pathogen *Legionella pneumophila* directly accesses the labile cytoplasmic Fe^{II} pool of the host cell by inserting an Fe^{II} selective transmembrane importer into the vacuolar membrane; once enriched inside the vacuole, Fe^{II} is then likely imported by the Feo transporter (Section 4.2).[72]

4.2 Feo system for Fe^{II} uptake

The major bacterial system that transports Fe^{II} across the cytoplasmic membrane is the Feo system (Figure 5A).[73] The canonical *feo* operon is comprised of three genes, named *feoABC* and is Fur regulated; however, the core transporter is FeoAB since FeoC is not encoded by the vast majority of *feo* operons.[74] FeoA is a small, cytoplasmic β-rich protein, while FeoB is a membrane protein typically comprised of three domains: an N-terminal cytoplasmic G protein domain, a GDP dissociation inhibitor domain (essentially a linker domain that connects the G domain to the transmembrane domain) and the transmembrane domain itself, thought to form the Fe^{II} transport channel.[73] FeoB is anticipated to couple

Fe^{II} transport with GTP hydrolysis with FeoA and FeoC functioning as accessory proteins in this process.[73–75]

Metal specificity of the Feo system.—FeoB was originally discovered by characterization of iron transport mutants that arose following the addition of antibiotics, which often generate intracellular ROS in the presence of Fe.[73, 76] Mutants that had low internal Fe and thus survived the treatment were isolated and *feo* locus was identified.[76] The specificity for Fe^{II} has generally been established by culturing siderophore biosynthesis deficient mutants under conditions where Fe^{II} speciation is favored. For example, in studies of *V. cholerae*, ascorbate added to the growth medium was found to stimulate ^{55}Fe uptake in a Feo-dependent manner, and thus it was concluded that Feo was specific for Fe^{II}.[77] However, FeoB homologs in *Porphyromonas gingivalis* and *Clostridium perfingens* impact Mn^{II} uptake as well, but the extent to which this characterizes other bacterial systems is not known.[78] Clearly, Fe-specific FeoB proteins from *E. coli* and *Klebsiella pneumoniae* are more closely related than are Fe-only vs. Fe-Mn FeoB2 transporters (88 vs. 31% sequence identity), but the origin for this apparent relaxed metal specificity in the latter is unknown. The metal specificity of Feo transporters, particularly those present as second copies in genomes, clearly warrants further investigation.

The Feo system and virulence.—FeoB is known to be crucial for the virulence of several microbial pathogens. *C. jejuni* and *Helicobacter pylori* are expected to encounter significant Fe^{II} in the relatively acidic and oxygen-limited conditions of the upper gastrointestinal tract,[74] and *feoB* mutants in *C. jejuni* and *H. pylori* have lower viability and decreased colonization efficiencies in the piglet intestine and the mouse stomach, respectively.[79] The same is true of *Salmonella* in infected macrophages.[80] The *feoB* transcript has been detected in *P. aeruginosa* extracted from the lungs of patients suffering from cystic fibrosis.[68a] FeoB may act in concert with other Fe uptake systems in some bacterial species such as *Yersinia pestis* and *Francisella tularensis* which demonstrate reduced viability only when a *feoB* deletion is accompanied by deletion of other Fe^{III} uptake systems.[81] Although most studies that investigate the impact of Feo system on bacterial virulence have focused on FeoB, a number of reports reveal that a *feoA* deletion can result in a significant reduction of Fe uptake and bacterial growth in several pathogens including *V. cholerae*, *Salmonella enterica* and *A. baumannii*, although the precise function of FeoA remains to be clarified.[82] For example, in *S. enterica*, both *feoA* and *feoB* deletion strains result in a ≈3-fold decrease in ferrous uptake, but only when other known Fe^{II} uptake systems are deleted. A *feoA-feoB* double mutant could be rescued from the defect in Fe^{II} uptake only by complementation of both genes, and FeoA interacts with FeoB *in vivo*, consistent with a functionally important interaction.[82b]

5. Mechanisms of Fe sequestration by the host

5.1 Fe^{III} sequestration by the host

The host deploys siderocalin (lipocalin 2) at sites of infection to sequester Fe^{III}-Ent and some other carboxymycobactin siderophores[83] thereby withholding Fe^{III} from the pathogen. Abundant host proteins lactoferrin and transferrin also play a role in sequestering

extracellular Fe^{III}. Siderocalin belongs to lipocalin family of proteins characterized by eight-stranded antiparallel β-barrel harboring a calyx where the Fe^{III}-siderophore complex binds (Figure 5B). Three positively charged side chains form a cation-π interaction with each of the three catechol rings of the *tris*-catecholate siderophore and there are additional hydrogen bonding interactions involving a highly conserved tyrosine residue (Figure 5B).[84] In the ongoing "arms race" for Fe in the infected host,[58, 85] some bacteria have adapted to siderocalin-mediated Fe^{III}-Ent sequestration by synthesizing modified Ent molecules that cannot be bound by siderocalin, and are therefore designated stealth siderophores (Figure 1). These include salmochelin, which is essentially Ent derivatized with a bulky C-linked glucose substituent on the catechol moieties, and petrobactin, produced by *Bacillus anthracis*, which incorporates a 3,4 catechol instead of 2,3 catechol characteristic of all other catecholate family siderophores (see Figure 2A).[83]

5.2 Fe^{II} sequestration by the host

Although originally described as playing a role in manganese and zinc restriction at sites of infection,[86] the multi-metal withholding protein calprotectin (Figure 5C) is now believed to play an important role in Fe^{II} sequestration,[87] certainly in liquid culture-based assays, [4b, 88] and perhaps at the host-pathogen interface. Secreted by neutrophils and highly abundant at infection sites, calprotectin is a heterotetramer of two S100 family proteins (S100A8 and S100A9; $\alpha_2\beta_2$) (Figure 5C).[89] Two distinct metal coordination sites present at its αβ heterodimer interface exhibit distinct metal specificities and are strongly activated to coordinate transition metals by Ca^{II}, which is abundant in the extracellular environment. [90] The His_3Asp site 1 binds Zn^{II} with picomolar affinity and is generally conserved in other S100-family metal-chelating proteins. The His_6 site 2, on the other hand, is a "jack of all trades" and coordinates Fe^{II}, Mn^{II}, Zn^{II} and Ni^{II} to form what are believed to be isostructural, His_6 octahedral coordination complexes[91] of high thermodynamic and/or kinetic stability.[92] Fe^{II} binding by calprotectin shifts the redox speciation of Fe from Fe^{III} to Fe^{II} in an aerobic environment in the absence of a extracellular reductant,[93] and is capable of withholding Fe from a number of Gram-positive and Gram-negative bacteria including *P. aeruginosa, E. coli*, *S. enterica* and *K. pneumoniae*.[88] [4b, 88, 92]

6. Physiological adaptation to host-mediated Fe^{II} or Fe^{III} sequestration by the pathogen

A primary physiological response to severe Fe restriction is induction of the Fur regulon. Fur and other Fe-sensing transcriptional regulators are global regulators in many organisms, and thus goes well beyond the regulation of genes encoding Fe acquisition systems to a significant re-programming of cellular metabolism. An important mechanistic feature of this re-programming is the expression of one or more Fur-regulated small regulatory RNAs (sRNAs) that inhibit the synthesis of non-essential Fe requiring proteins and enzymes, which for *P. aeruginosa* is important in murine lung infections.[6b, 7, 94] This results in post-transcriptional downregulation of genes that encode for cell-abundant Fe enzymes, including succinate dehydrogenase and cytochromes, which optimizes use of scarce bioavailable iron to metallate essential enzymes, a prioritization mechanism termed Fe sparing.[95] In *B. subtilis*, sRNA-regulated Fe sparing is effectively an adaptation of "last resort" and is within

the final “wave” of Fur-induced derepression observed upon increasingly severe degrees of Fe limitation.[6a] Some other effects include repression of chemotaxis and motility genes that has been observed during Fe limitation in *A. baumannii*.[96]

A major metabolic shift that may well represent a widespread, “frontline” adaptive response to Fe restriction is a shift toward flavin-anchored metabolic pathways, and away from a reliance on Fe-S-containing ferredoxins as cellular reductants. As ferredoxins and flavodoxins possess similar reduction potentials, flavodoxins are capable of substituting for ferredoxin as electron carriers (reductants) under conditions of Fe limitation, even for highly specific transformations including dehydration reactions in *Acidaminococcus fermentans*[97] and the desaturation of fatty acids in *B. subtilis*.[98] Indeed, genes encoding flavodoxins are highly transcriptionally induced under Fe limitation in *Clostridium acetobutylicum*[99] and in the strict anaerobe and intestinal pathogen, *Clostridium difficile*.[100] In many species of bacteria and algae, flavodoxin protein levels are detected only under conditions of Fe limitation [98c] and this is true for the flavodoxin WrbA in *A. baumannii* as well;[4b] indeed, the ratio of cellular flavodoxin to ferredoxin is often considered a benchmark for Fe limitation in marine phytoplankton.[101] In order to fully activate this metabolic switch, a concomitant prioritization of flavin biosynthesis must occur, which can result in an accumulation of riboflavin in the culture supernatant.[99, 102] The regulatory mechanisms that drive this switch, however, likely differ among organisms. In some bacteria, riboflavin biosynthesis is directly Fur-regulated, [95, 99, 102–103] while in others, this adaptive response to Fe limitation is not so straightforward.[104]

Recent work reveals that calprotectin induces an overlapping Zn^{II} and Fe starvation response in *A. baumannii*, as determined by changes in both the transcriptome and the soluble proteome.[4b] These changes include transcriptional upregulation of the Fe^{II} importer FeoAB, several Fe^{III} siderophore OM receptors, acinetobactin biosynthetic proteins and decreased cellular abundance of a number of Fe proteins, including the hemoprotein cytochrome b_{562} and the major ferredoxin FdxB, alongside induction of the entire Zur regulon.[4b, 105] More importantly, these studies uncover a strongly integrated coupling of extreme Fe/Zn^{II} restriction and *de novo* riboflavin biosynthesis and perhaps other metabolic processes. To illustrate, the first enzyme in the convergent *de novo* flavin biosynthesis pathway is encoded by *ribB* which catalyzes the conversion of ribulose-5-phosphate to 3,4-dihydroxy-2-butanone 4-phosphate (DHBP) (DHBP synthase or RibB). In *A. baumannii*, a novel RibB fusion protein, RibBX, an active DHBPS, becomes detectable only under conditions of calprotectin stress; RibBX appears to enhance flavin biosynthesis by dramatically increasing the flavin toxicity “set-point” thus working around riboflavin-sensing riboswitch-mediated inhibition of authentic *ribB* expression.[4b] Furthermore, the Zur-inducible, candidate Zn^{II} metallochaperone ZigA[106] becomes cell-abundant under calprotectin stress, and a calprotectin-treated Δ*zigA A. baumannii* strain grows poorly and fails to sustain cellular flavin levels. Riboflavin supplementation partially rescues this phenotype. How ZigA sustains flavin biosynthesis in *A. baumannii* is unknown, but it is known that this pathway harbors two obligate Zn^{II} metalloenzymes, including the rate-determining enzyme, GTP cyclohydrolase II, encoded by *ribA*. It is interesting to note that the Zur-regulated ZigA homolog in *Bacillus subtilis*, ZagA, appears to sustain folate

biosynthesis under conditions of zinc restriction, by interacting with an obligatory Zn^{II} metalloenzyme GTP cyclohydrolase I, encoded by *folE*.[107]

Detailed studies like these raise the interesting possibility that in some microbial pathogens, the adaptive response to Zn^{II} limitation may be more strongly interwoven with the Fe-limitation response than previously anticipated,[108] since both stresses may be present concurrently in the host, particularly for extracellular pathogens, and therefore potentially negatively impacts nearly the entire metalloproteome. For example, the biosynthesis of the broad-spectrum transition-metal binding metallophore, staphylopine, in *Staphylococcus aureus*, is under dual transcriptional control by both Fur and Zur.[108] In addition, low Zn or low Fe jointly regulate the expression of a "hybrid" ABC-family metal transporter in *Corynebacterium diptheriae*.[109] Detailed investigations of low Zn-low Fe crosstalk should yield new insights in a systems-level response to co-incident host-derived Zn^{II} and Fe restriction during infections.

7. Concluding Remarks

In this Concepts report, we highlight new "variations on a theme" of pathogen Fe acquisition that diverge from the well-studied *E. coli* Fe^{III}-Ent model. We place these new studies in the context of recent observations that begin to challenge the Fe^{III}-centric view of Fe acquisition at even aerobic or oxic/anoxic interfacial sites of infection, that collectively suggest an important role for Fe^{II} acquisition by the pathogen in these niches. In these sites, calprotectin and perhaps other small molecule divalent metal-specific chelators yet to be discovered, *e.g.*, those analogous to the broad-spectrum nicotianamine-like metallophores, staphylopine and pseudopaline from *S. aureus* and *P. aeruginosa*, respectively,[110] may well function here. Staphylopine biosynthesis, efflux, metal capture, and holo metallophore uptake precisely parallel the Fe^{III}-siderophore systems described here (Figures 3–4).[111] Staphylopine, whose biosynthesis is strongly induced by calprotectin stress,[112] does in fact bind Fe^{II} but with a stability constant (log K_{Fe} 12.1) nearly 1000-fold weaker than Zn^{II} (log K_{Zn} 15.0).[110a] Consistent with this, staphylopine outcompetes calprotectin for nutrient Zn^{II} in *S. aureus* during infections.[111a] and the closely related pseudopaline functions to capture Zn^{II} in *P. aeruginosa* cultures.[110b] It is interesting to note, in the context of a continuously evolving host-pathogen "fight over metals",[58] that an OM-localized calprotectin receptor in *N. gonorrhoeae* is capable of hijacking Zn^{II} from calprotectin for use as a nutritional source.[113] Although characterized in Zn^{II} uptake, Fe^{II}-bound calprotectin might function in this way as well.

Multi-modal mass spectrometry-based imaging approaches will continue to play an important role in future efforts to detect and quantify the chemical constituents linked to transition metal (Fe, Zn) speciation at tissue sites of infection, particularly important in the context of polymicrobial communities.[114] Such an approach was recently used to map the tissue distribution of siderophores in animals infected with *S. aureus* as well as for the detection of secreted proteins, *e.g.*, calprotectin,[86a, 115] at the host-pathogen interface.[52] Detailed mass spectrometry analysis of a pathogen secretome *ex vivo* or from infected animals may also be leveraged for the discovery of new metallophore-like small molecules bound to their biologically relevant metals, *e.g.*, as previously illustrated for the virulence-

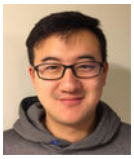
associated metallophore yersiniabactin in uropathogenic *E. coli* infections of the urinary tract,[116] or extracellular ferric reductases that could be involved in enhancing Fe^{II} bioavailability at sites of infection. The “arms race” for Fe[58] in the context of broad host-mediated transition metal restriction continues unabated and new discoveries await.

Acknowledgements

We gratefully acknowledge financial support from the US National Institutes of Health (NIH grants R35 GM118157 and R01 AI101171) and from Indiana University.

Biographical Sketches

Prof. David Giedroc obtained his Ph.D. under the supervision of J. David Puett at Vanderbilt University. He then carried out postdoctoral research with the late Joseph E. Colman of Yale University, a pioneer in the field of bioinorganic chemistry and enzymology of zinc metalloenzymes. He then established his own research group, first at Texas A&M University (1988–2007), and then at Indiana University in Bloomington, where he is the Lilly Chemistry Alumni Distinguished Professor in the Department of Chemistry. His group takes a problem-oriented, multidisciplinary approach to understanding the physical and chemical biology of transition metal homeostasis and more recently, reactive sulfur species and hydrogen sulfide homeostasis, in bacterial cells. He is Chair of the Editorial Board of *Metallomics*, a publication of the Royal Society of Chemistry (UK), and a Fellow of the American Association for the Advancement of Science (AAAS), the Royal Society of Chemistry and the American Academy of Microbiology.



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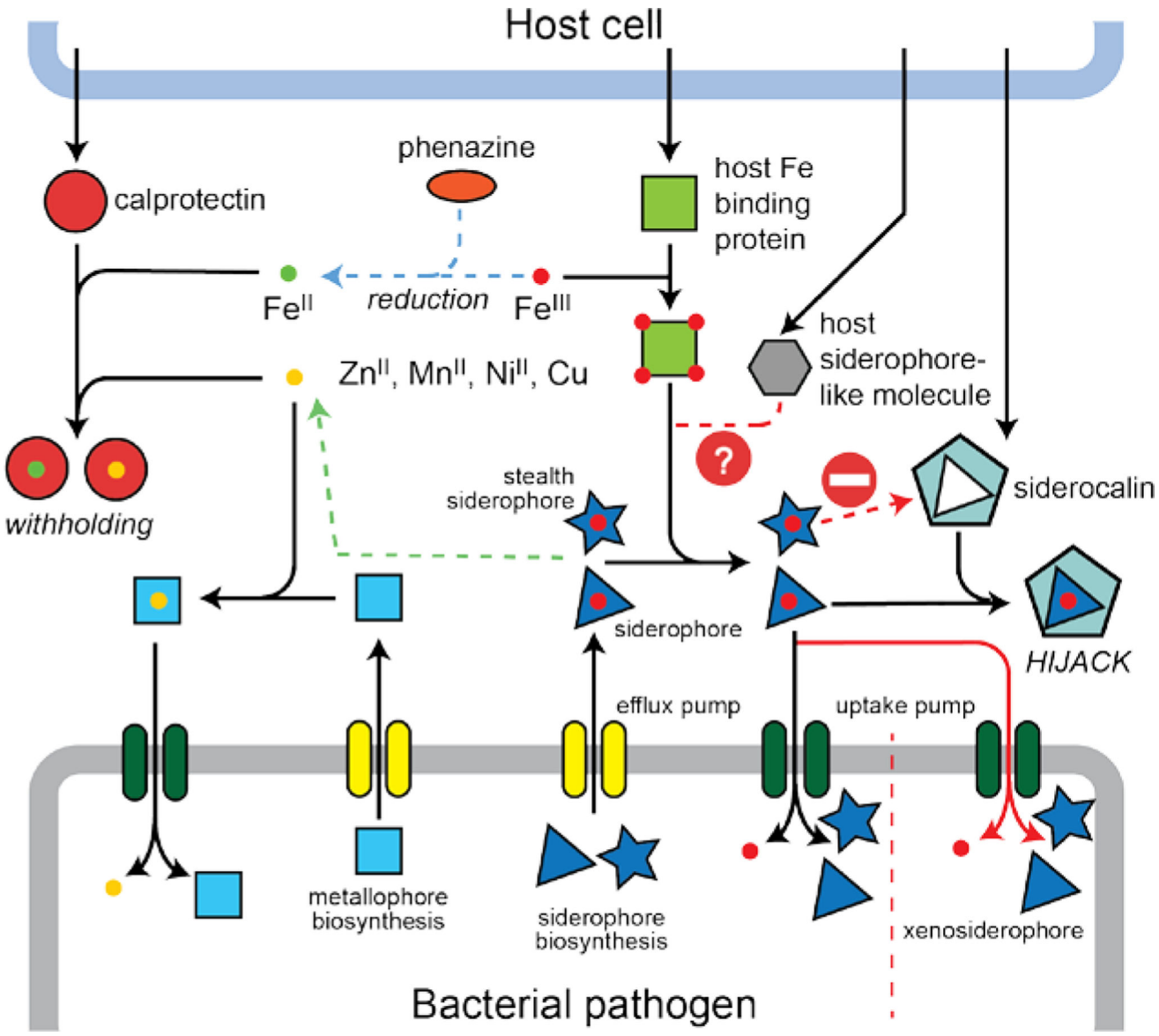

Figure 1.

A schematic overview of the fight over nutrient Fe at the host (*top*)-bacterial pathogen (*bottom*) interface during infections. The *green* arrow is meant to indicate that a previously characterized Fe^{III}-siderophore, *e.g.*, yersiniobactin, from *Yersinia* spp. and uropathogenic *E. coli*, is capable of binding other transition metals (*yellow* sphere) in infected animals.[116a, 116c] A host siderophore-like molecule is the simple catecholamine, norepinephrine (NE) (Figure 2A) that is proposed to strip Fe^{III} from transferrin in redox-dependent manner.[59]

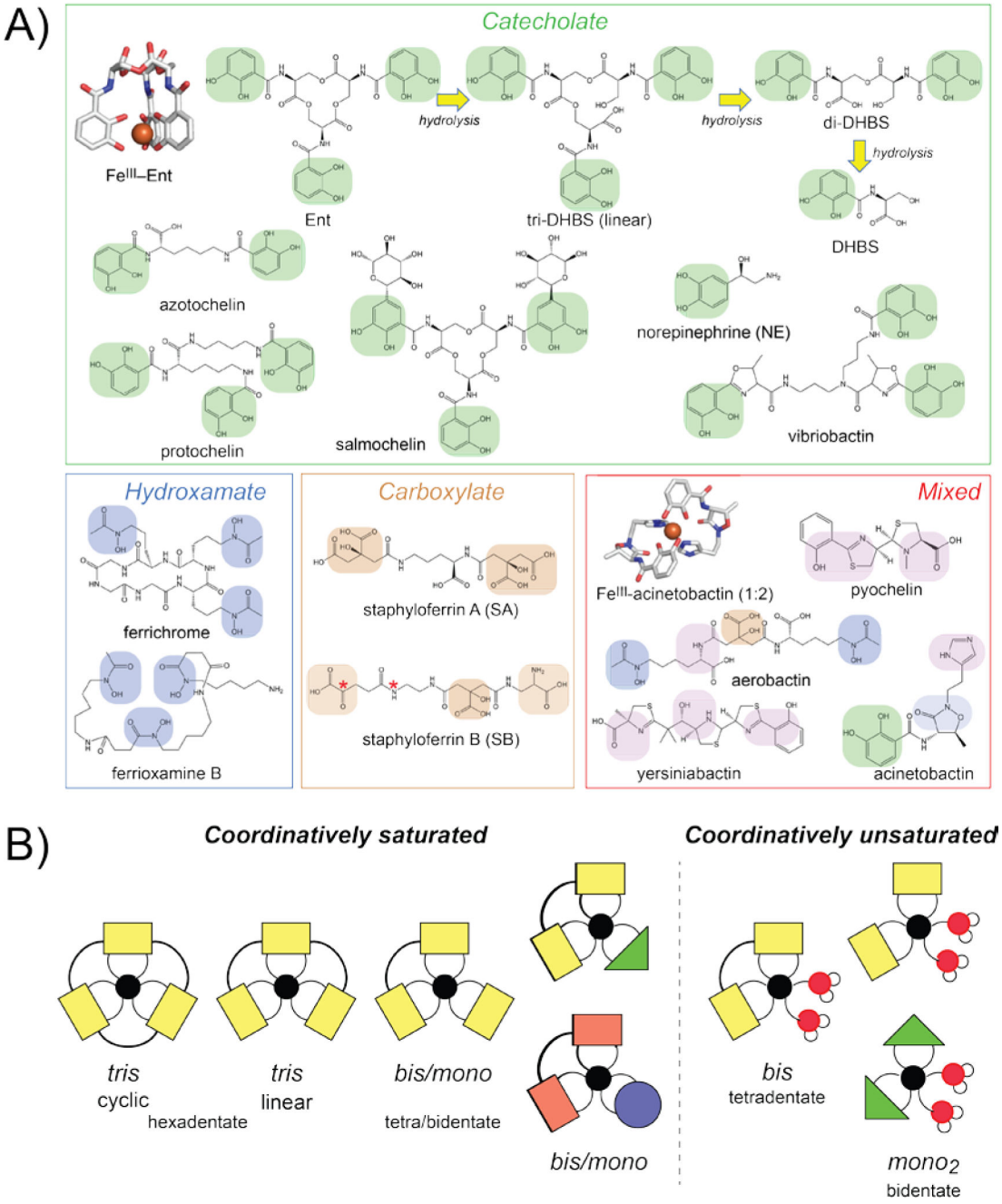

Figure 2.

A) Chemical structures of the four major classes of Fe^{III} siderophores, including the Ent hydrolysis products (*top*). This table is not meant to be exhaustive, but instead provides a snapshot of the structural diversity of the Fe^{III}-chelating small molecules discussed in the text. The three-dimensional structures of the Fe^{III}-Ent[26a] (pdb 6q5e; *P. aeruginosa* PfeA, see Figure 3B) and Fe^{III}-acinetobactin[38] (pdb 6mfl; *A. baumannii* BauB, the SBP) are shown, derived from protein-ligand complexes. The α-hydroxy carboxylate and α-amino carboxylate bidentate chelating moieties of the citrate-derived polycarboxylate siderophores SA and SB are highlighted in *brown*. *Red asterisks*, although the linear structure of the SB is

shown, these two atoms are known to cyclize to form a hemiaminal and resulting α-hydroxy carboxylate moiety in the active molecule.[31c] B) Cartoon illustrations of coordinatively saturated and coordinatively unsaturated Fe^{III}-siderophore complexes. The different shapes and colors represent distinct bidentate Fe^{III}-chelating (*e.g.*, catechol, hydroxamate, carboxylate) moieties. *Thick* lines, covalent connection of the component bidentate coordinating units; *thin* lines, coordination bonds to the Fe^{III} atom (*black* filled circle). *Red* filled circle symbol, solvent water molecule.

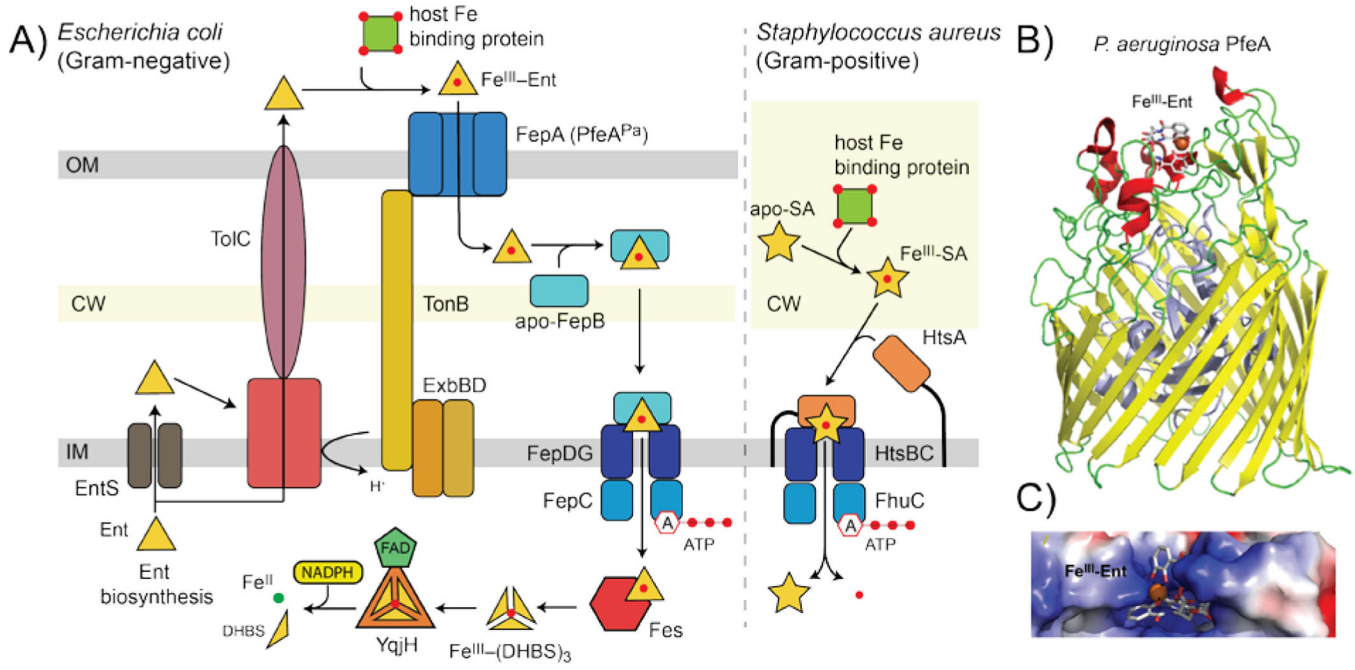

Figure 3.

A) An expanded view of Fe^{III}-Ent uptake (*left*) and Fe^{III}-staphyloferrin A (SA)[30] (*right*) as representative of steps involved in Fe^{III}-*tris*-catecholate uptake by Gram-negative bacteria and Fe^{III}-polycarboxylate siderophore uptake in Gram-positive pathogens, respectively. Siderophore biosynthesis is not shown. Although not discussed here, TolC is a periplasm-spanning protein required to pump apo-Ent and presumably other apo-siderophores into the extracellular space.[117] B) Ribbon representation of the structure of the OM β-barrel receptor *P. aeruginosa* PfeA bound to Fe^{III}-Ent (pdb 6q5c).[26a] C) Expanded view of the favorable electrostatics in the binding pocket for Fe^{III}-Ent.

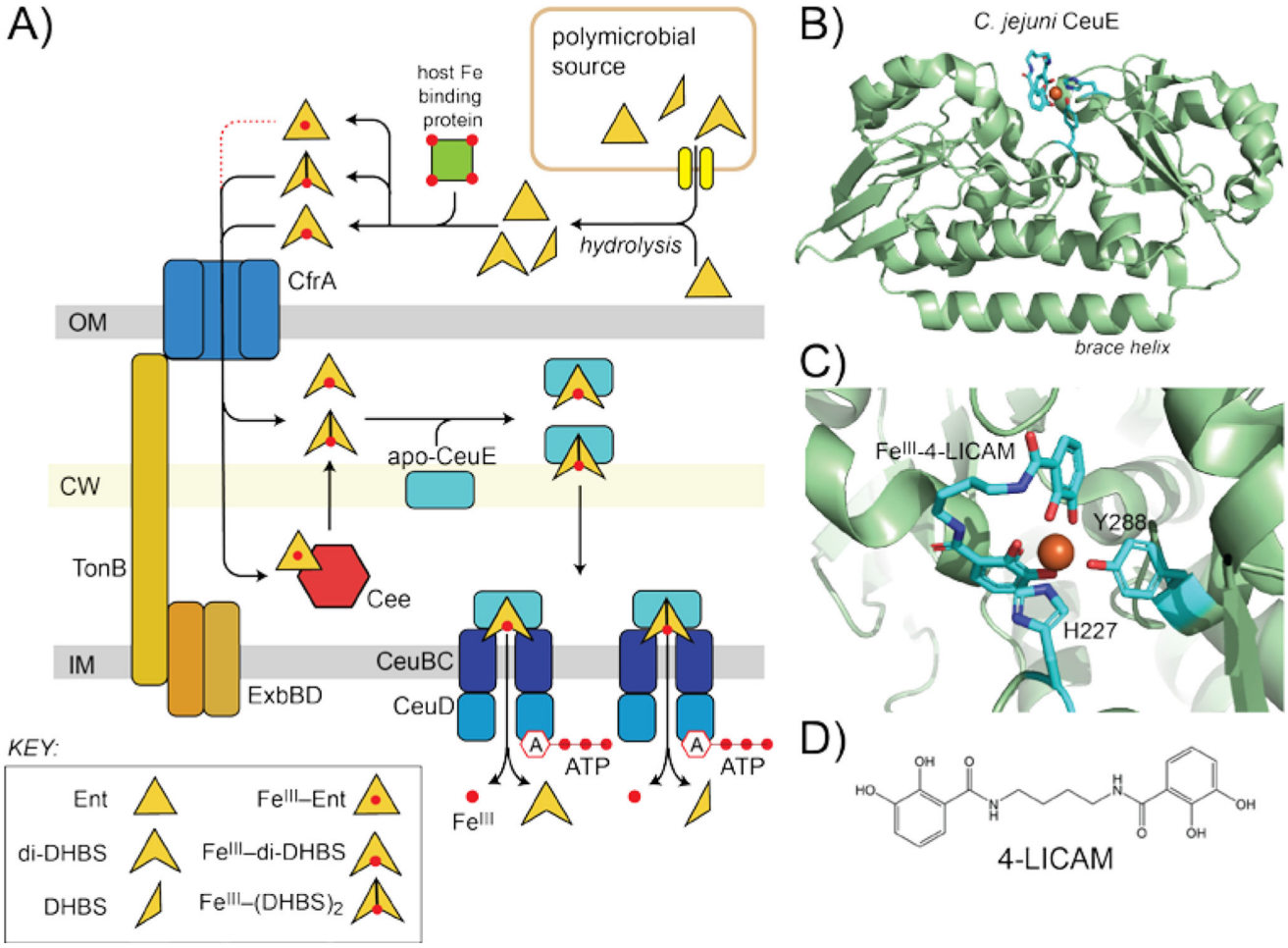

Figure 4.

A pictorial representation of the uptake of enterobactin (Ent) hydrolysis products by Ceu system in *C. jejuni*. A) Overall process. B) Ribbon representation of the structure of the Fe^{III}-4-LICAM complexed to the SPB CeuE (pdb 5a1j).[65] C) Close-up of the first coordination sphere around the Fe^{III}, with CeuE-derived ligands H227 and Y288 shown coordinating the metal. D) Chemical structure of the synthetic tetradentate *bis*-catechol, 4-LICAM.[65]

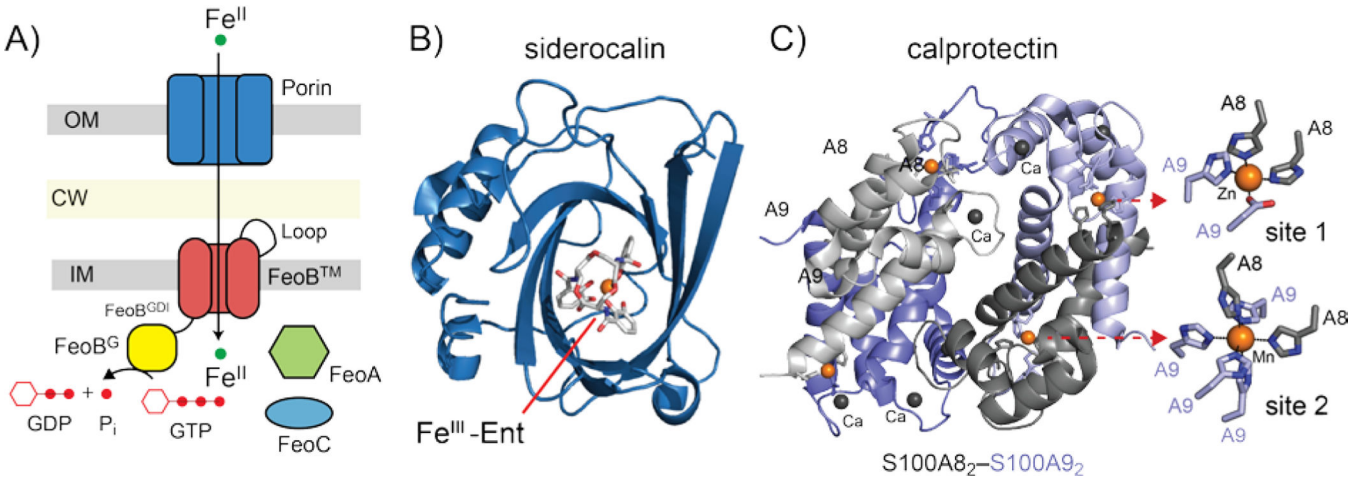

Figure 5.

A) Schematic illustration of Fe^{II} FeoB uptake system, with the three domains of FeoB indicated. See text for details. B, C) Host-derived weapons that limit the bioavailability of Fe^{III} and Fe^{II}, respectively, at infection sites. B) K125A derivative of human siderocalin bound to Fe^{III}-Ent (pdb 3cmp).[118] Fe^{III}-Ent binds to positively charged calyx in the center of the β-barrel where favorable electrostatics govern the affinity of the complex.[119] C) Calprotectin. Ribbon representation of the structure of the $S100A8_2$–$S100A9_2$ heterotetramer is shown (pdb 4ggf), with an expanded view of the site 1 and site 2 transition metal coordination sites, bound to Zn^{II} and Mn^{II} respectively, in this structure (*right*).[89] The locations of the four Ca^{II} binding sites are also shown.