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Microbial Iron Acquisition: Marine and Terrestrial Siderophores

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1. Introduction

The vast majority of bacteria require iron for growth.[1,2] Iron is an essential element required for key biological processes including amino acid synthesis, oxygen transport, respiration, nitrogen fixation, methanogenesis, the citric acid cycle, photosynthesis and DNA biosynthesis. However, obtaining iron presents challenges for the majority of microorganisms. While iron is the fourth most abundant transition metal in the Earth's crust, the insolubility of iron(III) [K_{sp} of $Fe(OH)_3 = 10^{-39}$] at physiological pH in aerobic environments severely limits the availability of this essential nutrient. Pathogenic and marine bacteria face similar challenges for obtaining iron because both live in very low iron environments. Bacteria typically require micromolar levels of total iron for growth, yet the iron concentration in the surface waters of the oceans is only 0.01-2 nM.[3-7] In humans cellular iron is also very low and is sequestered by lactoferrin, transferrin, and ferritin as a primary defense mechanism at the onset of infection.[8] Given its cellular importance, it is not surprising that microbes have evolved multiple pathways designed to extract iron from their surrounding environments, tailored to the molecular constraints of the iron pool (Figure 1).

In this review the general pathways by which bacteria acquire iron are considered first as an overview to illustrate the singular importance of iron for microbial growth. The focus of this review is on siderophore-mediated iron uptake, particularly structural characteristics of marine siderophores and the reactivity that these characteristics confer. Relatively little is known about marine microbial iron transport compared to that for terrestrial and pathogenic microbes, yet comparison of the structures and reactivity may hint at the biological advantage that these structural traits confer to marine microbes and very possibly provide insights to siderophore-mediated iron uptake in some pathogens.

2. Iron acquisition pathways

2.1. Ferric iron uptake

2.1.1. Siderophore-mediated iron uptake—Bacteria growing under aerobic conditions in the absence of readily available iron often produce siderophores to solubilize, capture, and deliver Fe(III) to the cells. Several hundred structures of terrestrial siderophores are known, although relatively few structures of marine siderophores have been identified. Gram-negative bacteria have outer membrane receptor proteins which recognize specific iron(III)-siderophore complexes at the cell surface. Ferric siderophore complexes are actively transported across cell membranes though an energy-dependent system consisting of the outer membrane siderophore receptor proteins, periplasmic binding proteins and inner membrane transport proteins.[1,2] In Gram-positive bacteria, ferric siderophore complexes are also recognized by specific membrane receptor proteins and transported into the cytoplasm by ABC-type transport proteins.[9]

Relatively few outer membrane siderophore receptor proteins have been crystallized, however several outer membrane siderophore receptor systems have been identified in numerous bacteria through genetic analyses. The high-resolution structures available, FepA (ferric

enterobactin permease),[10] FhuA (ferric hydroxamate uptake),[11,12] FecA (ferric citrate),[13,14] FptA (ferric pyochelin transport),[15] FpvA (ferric pyoverdin),[16-18] as well as molecular models of the outer membrane proteins FvtA (ferric vanchrobactin transport),[19] and FatA (ferric anguibactin transport)[20] show many structural similarities between siderophore receptors.

The structural core of these receptors is comprised of two domains, a C-terminal domain that includes a membrane-spanning, 22-strand, anti-parallel β-barrel with large extracellular loops extending outside the membrane bilayer, and an N-terminal domain which fills the interior of the barrel, making a plug. This plug has a four-stranded β-sheet domain and interspersed α-helices and loops, including two loops that extend beyond the membrane bilayer interface towards the extracellular loops of the barrel, forming the region involved in siderophore recognition and binding.[21] The largest difference between the receptors is the nature of this site, which is specific for siderophores that differ in structure and charge.

Once the ferric siderophore binds to the outer membrane receptor protein, the outer membrane protein-ferric siderophore complex interacts with the inner membrane protein TonB (i.e., transport of iron) at a conserved sequence of five amino acids near the N terminus called the "TonB box" (TXXV[S/T]), where X is a hydrophobic residue. The substrate-bound outer membrane receptor then undergoes an energy-driven conformational change coupled to the proton gradient across the inner membrane,[22-26] leading to the transport of the ferric siderophore complex across the membrane and release into the periplasm. The energy transfer from the inner membrane to the outer membrane is facilitated by a complex of TonB with proteins ExbB and ExbD which are also anchored in the inner membrane.[27]

After the ferric siderophore is released to the periplasmic space, it is bound by a high affinity periplasmic binding protein (e.g. FhuD K_d 0.1 μM for the ferric ferrichrome siderophore complex),[29] which escorts the ferric siderophore complex to the cytoplasmic membrane. Binding of the ferric siderophore complex to the periplasmic binding protein also prevents the transport of the ferric siderophore complex back out across the outer membrane.

Ferric siderophore complexes are transported across the cytoplasmic membrane by an ATP binding cassette (ABC) transporter protein complex which couples the hydrolysis of ATP to siderophore transport. The ferric siderophore complex passes through the ABC transporter via a channel formed of two transmembrane domains while two nucleotide domains hydrolyze ATP.[21] ATP hydrolysis induces a conformational change in the ABC transporter protein allowing the ferric siderophore to be transported into the cytoplasm.[21]

2.1.2. Heme-mediated iron uptake—Heme is an important iron source for certain bacteria.[30] Bacteria can acquire iron from heme either by expressing outer membrane receptors and transport proteins specific for heme and/or secreting hemophores, specialized bacterial proteins able to sequester heme from diverse environments and deliver it to its specific outer membrane receptor.[31,32]

The crystal structure of the *Serratia marcescens* hemophore HasA bound to its outermembrane receptor protein HasR has recently been reported (Figure 4).[33]

HasA is a 19 KDa monomer protein composed of a β sheet and a layer of 4 α-helices. Heme is bound within two loops located at the layer interface by axial coordination with His-32 and Tyr-75.[34] HasR is similar in structure to ferric siderophore outer membrane receptors. In addition to binding hemophore HasA, HasR can also bind heme and hemoglobin.[35] The mechanism of heme acquisition and transport in bacteria is similar to the ferric siderophore system. Heme transport through the membrane requires the proton motive force of the cell and energy provided by the cytoplasmic membrane proteins TonB, ExbB and ExbD.[36] The fate of

heme once it reaches the cytoplasm is less well known. However, evidence suggests that iron is released from heme by heme oxygenase by oxidation and degradation of the tetrapyrrole ring.[37] Recently oceanic bacteria have now been shown to have a heme-mediated iron uptake system (see below, Section 5.)

2.1.3. Transferrin- and lactoferrin- mediated iron uptake—Select Gram negative bacteria, specifically *Haemophilus influenzae*, *Neisseria meningitides*, and *Actinobacillus (Haemophilus) pleuropneumoniae* are able to obtain iron directly from their host's iron transport proteins by expressing outer membrane receptor proteins for lactoferrin and transferrin.[38,39,40] Iron is removed from the host's iron binding protein by direct contact between the bacterial cell surface and the host's iron binding protein. Binding of transferrin and lactoferrin is species specific, e.g. only human forms of these proteins are recognized and used as iron sources by the pathogenic *Neisseria* spp. whereas the pathogen *Actinobacillus (Haemophilus) pleuropneumoniae* is able to bind and use pig, but not human, transferrin as an iron source.[39,41] Two proteins, TbpA and TbpB work together as the functional transferrin receptor.[42] TbpA serves as the outer membrane pore through which iron, removed from the host's transferrin, enters the bacterial cell periplasm. TbpB is thought to be a member of the family of TonB-dependent receptors, analogous to the ferric siderophore receptors, and most likely increases the efficiency of the functional receptor.[43] Much less is known about iron acquisition in Gram positive bacteria than in Gram negative bacteria, however a transferrin receptor, Tpn, has been identified in certain species of Gram positive *Staphylococci*.[44-46]

2.1.4. Ferric binding protein (Fbp)—While several mechanisms of iron acquisition pathways (e.g. siderophore, heme, and transferrin/lactoferrin uptake) have been identified at the cell surface, the fate of iron once released into the periplasm lies with a single ferric binding protein (Fbp). Fbp serves to shuttle iron from the inside of the outer membrane across the periplasm to the cytoplasmic membrane. The first crystal structure of Fbp was characterized from *Haemophilus influenza* (hFbp) (Figure 5).[47]

Fbp is structurally related to transferrin proteins, which consist of two lobes connected by a 'hinge' of two antiparallel beta stranded sheets. Each lobe contains a single Fe^{3+} ion, which in the single-lobed hFbp is coordinated by two tyrosines, a histidine, a glutamic acid, a water molecule and an exogeneous phosphate anion forming an octahedral geometry. Given the diverse nature of the periplasm, the exact nature of the exogenous coordinating anion has been questioned and numerous studies have been devoted to examining the role of the exogenous anion in the overall function of Fbp.[48-51]

2.2. Ferrous iron uptake

While mechanisms of ferric iron uptake in bacteria have been widely studied, the mechanism of ferrous iron uptake is less well understood. Ferrous iron is relatively more soluble at neutral pH compared to ferric iron and therefore may be transported more readily across the outer membrane, however ferrous iron will only predominate over the ferric form under reducing or anaerobic conditions. Not surprisingly ferrous iron uptake (Feo) systems have been identified in bacteria which grow in anaerobic or in microaerophilic environments. The Feo system was first identified in *E. coli*, which helps maintain anaerobic conditions in the gut, by Hantke et al.[52] The *E. coli* Feo system has three major units: FeoA, a cytoplasmic protein with an SH3-like domain; FeoB, a cytoplasmic membrane protein with an N-terminus G-protein domain, and an integral membrane spanning C-terminus domain containing two gate motifs which function to transport the ferrous iron across the cytoplasmic membrane through an ATP/GTP-driven active transport process; and FeoC, a small protein functioning as an [Fe-S]-dependent translational receptor.[53] In addition to transporting ferrous ion, there is limited evidence that

some bacteria are capable of actively reducing ferric iron through the production of extracellular reductases.[54,55]

2.3. Regulation of siderophore production

2.3.1. Ferric uptake regulator (Fur)—While the majority of microbes require iron for growth, excess iron can have toxic cellular effects (e.g. Haber-Weiss reactions and Fenton reactions) and regulation of iron uptake plays a crucial role in microbial survival. The ferric uptake regulator (Fur) protein regulates iron homeostasis in bacteria. During conditions of sufficient iron concentrations within a bacterial cell, Fur coordinates Fe(II) which enables it to bind to a specific DNA sequence, known as the Fur box, and thus repress transcription of genes controlling iron uptake processes, including siderophore biosynthesis and ferric siderophore outer membrane receptor protein expression.[56]

Crystal structures have been determined for Fur proteins from *Pseudomonas aeruginosa* (*Pa*Fur),[57] *E. coli* (*Ec*Fur) (truncated protein (Fur-(1-82)),[58] and most recently *Vibrio cholerae* (*Vc*Fur) (Figure 6).[59]

Fur is a dimeric metalloprotein consisting of two ~17 kD monomer units. The N-terminal domain binds DNA and the C-terminal domain is involved in dimerization. Each monomer unit (~17 kD) contains two zinc binding sites: one site is located in the DNA binding domain and the other is located in the dimerization domain. In *Vc*Fur the coordination geometry of both zinc ions is tetrahedral.[59] In contrast, the crystal structure of *Pc*Fur revealed that the zinc ion in the DNA binding domain was coordinated by a fifth water ligand and therefore the coordination sphere is described as a distorted octahedron.[57] The zinc ion in the dimerization domain is coordinated by four conserved amino acid residues His 87(86), Asp 89(88), Glu 108 (107) and His 125(124) (The residue numbers are from *Vc*Fur with numbers from *Pc*Fur in parentheses). Similarly, the zinc ion located in the area that connects the DNA binding region to the dimerization region is coordinated by His 33(32), Glu 81(80), His 90(89), and His 88 (in *Pv*Fur Glu 100 replaces His 88).

Approximately 90 genes are subject to transcriptional repression by iron in bacterial strains containing Fur.[56] The majority of the regulated genes encode metal acquisition functions, however numerous studies have revealed that many other processes including respiration, chemotaxis, glycolysis, DNA synthesis and the citric acid cycle, are regulated by Fur. Fur is therefore considered a global regulator, further highlighting the importance of the role of iron in bacteria.

2.3.2. Regulation of siderophore production by quorum sensing—In addition to Fur regulation, siderophore production has been found to be regulated by quorum sensing in select bacteria. Quorum sensing is a cell-density dependent process used by bacteria to regulate various tasks. Bacteria produce and respond to small signaling molecules, such as acyl homoserine lactones (HSLs). Acyl-HSLs diffuse in, out and between bacterial cells. An increase in bacterial population results in an increase of acyl-HSLs. The acyl-HSLs then bind to specific receptor proteins, which interact with the bacterial DNA, triggering a phenotype response. Examples of phenotypes regulated by quorum sensing include: biofilm formation, swarming motility, bioluminescence, and antibiotic and toxin production.[60-62]

Quorum sensing systems in many pathogenic bacteria including *Pseudomonas aeruginosa*, *Vibrio harveyi*, *Vibrio alginolyticus*, *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Burkholderia cepacia*, are reported to influence siderophore production.[63-69] *P. aeruginosa* produces two siderophores, pyochelin[70] and pyoveridine.[71] Pyoverdine production decreases in *P. aeruginosa* mutants lacking the quorum sensing regulator *lasR*, although the affect on pyochelin biosynthesis was not reported.[63,72] While decreased

siderophore production in bacteria lacking quorum sensing regulators appears to be more common, the opposite effect in *Burkholderia cepacia* was observed where mutants lacking the homologous quorum sensing regulator *cepR* had increased production of the siderophore ornibactin.[69] Additional studies are needed to further elucidate the regulatory mechanisms of quorum sensing on iron acquisition in bacteria.

3. Siderophores

3.1. Classification of siderophores

Common functional groups in siderophores that coordinate to Fe(III) include catechols, as in enterobactin; hydroxamic acids, as in the desferrioxamines; and α-hydroxycarboxylic acids, as in achromobactin (see structures below). Each of these functional groups is an OO′ donor and within these representative siderophores, the tris OO′ coordination results from three catecholates in enterobactin, three hydroxamates in the desferrioxamines, and three α-hydroxy carboxylates in achromobactin, although mixed functional group siderophores are more prevalent, as in aerobactin with two hydroxamates and one α-hydroxy carboxylate ligand.

3.1.1. Tris catecholate siderophores—Enterobactin, bacillibactin and salmochelin are all tris-catechol siderophores framed on a cyclic tri-ester scaffold of L-serine or L-threonine (Figure 7). Enterobactin, isolated from many different enteric and pathogenic bacteria including *E. coli*, is a cyclic trimer of 2,3-dihydroxybenzoyl-L-serine. Salmochelin, isolated from *Salmonella enterica*, and uropathogenic *E. coli*, [73,74] is a glucosylated derivative of enterobactin, in which up to two catechols contain a glucose at position C-5.[75,76] Bacillibactin, isolated from *Bacillus subtilis* and other *Bacilli* species, incorporates a cyclic triester scaffold of L-threonine. Each threonine amine is appended by glycine that is ligated by 2,3-dihydroxybenzoic acid. Thus the glycine spacers elongate the three chelating arms compared to enterobactin.[77]

The proton independent stability constant for the tris catecholato siderophores are remarkably large with Fe(enterobactin)$^{3-}$ at 10^{49},[78] and Fe(bacillibactin)$^{3-}$ at $10^{47.6}$.[77] In each case, Fe(III) is present in the high-spin electronic configuration. Surprisingly, a high resolution x-ray structure of the discrete Fe(enterobactin)$^{3-}$ compound has not yet been achieved, although an x-ray structure of Fe(enterobactin)$^{3-}$ bound to siderocalin protein has been reported at 2.4 Å resolution.[79,80] In addition, the crystal structure of the vanadium(IV)-enterobactin complex, (i.e., bare V(IV) without an oxo ligand), reveals a Δ-configuration at the V(IV) center.[81,82] The circular dichroism spectra of [Fe(III)(enterobactin)]$^{3-}$ as well as the substitution inert [Cr^{III}(enterobactin)]$^{3-}$ and [Rh^{III}(enterobactin)]$^{3-}$ complexes are consistent with the Δ right-handed propeller configuration.[81,83-85] In addition, while both enterobactin and bacillibactin are each tris catecholate siderophores deriving from a cyclic triester backbone of L-serine or L-threonine, respectively, the chirality at the metal center is opposite to each other. [86]

3.1.2. Tris hydroxamate siderophores—The ferrioxamines are a well-known group of tris-hydroxamate siderophores which are primarily assembled from alternating units of succinic acid and a monohydroxylated diamine, that is, N-hydroxy-cadaverine or N-hydroxy-putrescine (Figure 8). Representative examples of tris hydroxamate siderophores include desferrioxamines B, G and E, in which desferrioxamine E is the cyclic counterpart to the linear desferrioxamine G.[87] Desferrioxamine B (DFOB) is the drug Desferal used to treat iron overload disease. Upon Fe(III) coordination, the conformer of the hydroxamic acid group switches to the cis, Z configuration for bidentate coordination, whereas in the absence of metal coordination, it exists in the E as depicted in Figure 8.

3.1.3. α-Hydroxycarboxylate, carboxylate and mixed functional group siderophores—α-Hydroxycarboxylic acids are another good bidentate chelating group for

Fe(III) coordination. Achromobactin is a tris-α-hydroxycarboxylate siderophore whose biosynthesis has recently been reported (Figure 9).[88,89] Two α-hydroxycarboxylate groups in achromobactin derive from α-ketoglutarate, and the third α-hydroxycarboxylate comes from citric acid. Bis α-hydroxycarboxylic acid siderophores, such as vibrioferrin are comprised of one α-hydroxycarboxylate from citrate and one from α-ketoglutarate, whereas the α-hydroxycarboxylates in staphyloferrin and rhizoferrin come from two citrate groups. Surprisingly, vibrioferrin has been shown to bind boron stoichiometrically through the α-hydroxycarboxylic acid groups, with an appreciable stability constant, $10^{14.1}$.[90,91] Perhaps the simplest siderophore is citric acid.[92] It is believed to function in Fe(III) uptake as the bis-ferric-citrato complex, $(Fe\text{-}citrate)_2$, which is recognized by the outer membrane receptor protein, FecA.

Many siderophores contain more than one type of functional group moiety, such as aerobactin (Figure 9). In fact, the vast majority of siderophores are comprised of different types of bidentate donor ligands. The variety of this mix of chelating groups is evident in the siderophore structures presented below.

3.2. Marine Siderophores

While relatively few siderophore structures from marine bacteria are known compared to the numerous terrestrial siderophores, two structural features dominate the majority of the marine siderophores discovered to date (Figure 10).[93,94] So far, the majority of marine siderophores have been found to be produced as families of amphiphiles, composed of an iron(III)-binding head-group that is appended by one or two of a series of fatty acids.[93,95-99] The other structural feature is the presence of an α-hydroxycarboxylic acid moiety, in the form of β-hydroxyaspartic acid or citric acid, which are photoreactive when coordinated to Fe(III).[100-105] Many of the marine siderophores are both amphiphilic and photoreactive in their Fe(III)-coordinated state. Figure 10 shows the suites of marine amphiphilic siderophores reported so far.

3.2.1. Amphiphilic Siderophores—The suites of amphiphilic siderophores range from being quite hydrophobic, such as the amphibactins and ochrobactins, to rather hydrophilic, such as the loihichelins.[99] Variation in amphiphilicity arises based on differences in the head group composition relative to fatty acid chain length. The hydrophobicity of the amphibactins, with only four amino acids in the head group and comparatively longer fatty acid appendages (primarily C18 and C16), renders these siderophores cell-associated and in fact they are isolated by extraction from the bacterial pellet.[97] Similarly, the ochrobactins with a small citrate-based head group and two fatty acid appendages (C_8-C_{10}) are cell-associated and are isolated by extraction from the bacterial pellet.[96]

Even within one family of siderophores the amphiphilicity varies significantly as a result of fatty acid chain-length variations on a constant head group. The marinobactins with six amino acids in the head group are ligated predominantly with $C_{16:1}$ and $C_{16:0}$ fatty acids (i.e. marinobactins D and E, respectively), place them in the middle of the amphiphilic spectrum of these siderophores. Marinobactins A-E are isolated from the supernatant, however a small amount of marinobactin F with a $C_{18:1}$ fatty acid tail has been extracted from the bacterial cell mass indicating it is noticeably more hydrophobic.[108]

Marinobactin A-E partition into phospholipid membranes differentially according to the nature of the fatty acid appendage.[106] Apo-marinobactin E, with a C16:0 fatty acid, partitions the most, while introduction of a cis double bond, as in marinobactins D1 and D2, or reducing the fatty acid chain by two methylene units, as in marinobactin C, drops the extent of partitioning by an order of magnitude. The same trend occurs in the reduction in partitioning by 10-fold for marinobactins B and A, respectively relative to marinobactin C.[106] However, the ferric

marinobactin E complex partitions to a much smaller extent than apo-marinobactin E (i.e, 1.3×10^4 vs 6.3×10^5, respectively).[106]

The same trend in partitioning of amphiphilic siderophores, including the substantially reduced partitioning of Fe(III)-amphiphilic siderophore over the corresponding apo-siderophore is also observed with the ochrobactins.[96] That is, ochrobactin C with two C10:1 fatty acids partitions more than ochrobactin B, with one C10:1 and one C8:0 fatty acid. However, the ferric complexes of an alternate, photooxidized form of this siderophore (see section 3.2.2., below) partitions to nearly the same extent as the apo-ochrobactins B and C a result that is still under further investigation.[96]

A partial explanation for the decreased membrane partitioning of ferric ochrobactin versus apo-ochrobactin may come from consideration of the iron(III), gallium(III) and apo acinetoferrins (see structure below in Figure 21).[111] Acinetoferrin resembles the ochrobactins, with a citrate backbone and two acyl appendages, however the spacer between fatty acid and the terminal citrate carboxylates differs; the spacer is lysine in the ochrobactins, but 1,3-diaminopropane in acinetoferrin. However the OO′ donor groups are the same, with two hydroxamates and the α-hydroxycarboxylate of citrate. Apo acinetoferrin is reported to be considerably more hydrophobic than Fe(III)-acinetoferrin.[111] Molecular modeling shows that the two fatty acids are held in an antiparallel arrangement, splayed out at an approximated 130° angle in the Ga (III)-complex, whereas the fatty acids in apo-acinetoferrin are portrayed in a parallel fashion, poised for duo partitioning into a bilayer membrane.[111]

Other amphiphilic properties of the marinobactins that have been investigated, include self-assembly to form micelles as well as other vesicular structures both in the presence and absence of Fe(III) coordination.[98,107,109,110] The critical micelle concentration (CMC) of apo-M_E and Fe(III)-M_E are relatively low at ~ 50 μM and ~ 75 μM, respectively.[98] At concentrations above the CMC, apo-M_E aggregates to form spherical micelles (~ 4.6 nm in diameter) that decrease in size upon coordination of Fe(III) (~ 3.5 nm in diameter), as analyzed by small-angle neutron scattering (SANS). [107,109] The decrease in micelle diameter of Fe(III)-M_E is attributed to an increase in the head-group area relative to the lipid tail-volume (Figure 11), [112] which is consistent with molecular modeling results.[106]

In the presence of excess Fe(III), small angle neutron scattering data (SANS) is best fit by a mixed population of spherical micelles and large vesicles. [107] Dynamic light scattering (DLS) reveals vesicles that are ~190-200 nm in diameter. [107,109] Interestingly, addition of Zn(II), Cd (II), or La(III) to Fe(III)-M_E also induces vesicle formation to a greater extent than Fe(III). [109] A Bragg peak develops in the SANS profiles with increasing concentration of Zn(II), Cd (II), La(III) or excess Fe(III), consistent with formation of multilamellar vesicles (Figure 12).

The same micelle-to multilamellar vesicle transition is observed for Zn(II) addition to Fe(III)-M_B and Fe(III)-M_D.[109] As might be expected, the interlamellar repeat distance is smaller in these vesicles, ~5.0 and ~5.5 nm for Zn(II)-induced Fe(III)-M_B vesicles and Zn(II)-induced Fe(III)-M_D vesicles, respectively, compared to ~ 6.0 nm for the Zn(II)-induced Fe(III)-M_E vesicles. However, for vesicles to form, Fe(III) must be coordinated at the peptide head group; and Zn(II) addition to apo-M_E does not induce the micelle to vesicle transition.[109]

The terminal carboxylic acid of the marinobactins, which is not involved in coordination in the monomeric Fe(III)-M_E siderophore complex, is available for coordination to the added cations (Figure 13). EXAFS reveals that the terminal carboxylates of two Fe(III)-marinobactin complexes are cross linked through coordination by the Zn(II) and Cd(II) cations.[110] Zn(II), Cd(II), La(III), and Fe(III) form 2:1 ligand/metal coordination complexes with carboxylic acids such as acetic acid. [109,113] In contrast, metals that lack the preferential bis carboxylate

coordination do not induce this phase change, such as Ba(II) and Ca(II).[109,110,113] Moreover, as expected, addition of EDTA to the cation-induced vesicles distrupts the vesicles, presumably by coordination of the cross linking cation, Zn(II), Cd(II), La(III), and excess Fe(III).[109,110]

3.2.2. Photoreactive Ferric Siderophore Complexes

Citrate-containing siderophores: Ferric complexes of α-hydroxy carboxylic acid siderophores, including citric acid and β-hydroxyaspartic acid, are photoreactive (see Figure 14). The photoreactivity has been reported for the citrate-containing Fe(III)-aerobactin,[100] the Fe(III)-ochrobactins,[96] the Fe(III)-synechobactins,[95] and the Fe(III)-petrobactins (for the structure, see Figure 19, below), [103,104,114] as well as for ferric citrate complexes.[115] UV photolysis into the charge transfer band from the α-hydroxy carboxylate moiety to Fe(III) induces ligand oxidation and release of CO_2 along with reduction of Fe(III) to Fe(II).

The Fe(III)-aerobactin photoreaction has been studied most extensively, so far (Figure 14). Aerobactin is produced by a marine Vibrio species,[116] as well as by many other terrestrial and pathogenic bacteria. The photooxidation of the citrate backbone of aerobactin produces 3-ketoglutarate, as established by ^{1}H and ^{13}C NMR, mass spectral analyses and deuterium exchange in the photoproduct as a result of the keto-enol tautomerization (Figure 14).[100] The enolate form of the photoproduct prevails in water, which is also the form that coordinates Fe (III). The decrease of 46 mass units in the apo-photoproduct compared to apo-aerobactin results from loss of CO_2 and two protons. Perhaps most surprisingly, the affinity of the aerobactin photoproduct for Fe(III) is remarkably similar to that of aerobactin.[100,117]

UV photolysis of the ferric complexes of the ochrobactins (Figure 10),[96] the synechobactins (Figure 10),[95] and the petrobactins (structure shown in Figure 19, below)[118] produce the same conversion of the citrate backbone to 3-ketoglutarate and coordination of Fe(III) by the enolate form of 3-ketoglutarate in the photoproduct.

Photolysis of Fe(III) vibrioferrin (a marine siderophore produced by Marinobacter sp. strains DG870 and DG979),[119] however, is different because the two bidentate OO′ donor ligands derive from different kinds of α-hydroxycarboxylic acids, one from citrate and one from α-ketoglutarate.[120] The photoreaction leads to oxidation of the α-ketoglutarate α-hydroxycarboxylic acid center and not the citrate α-hydroxycarboxylic acid (Figure 15).

Ferric citrate complexes have been known for nearly a century to be photoreactive.[121,122] A dimeric ferric citrate complex, $Fe(III)_2\text{-}(cit)_2^{2-}$, [123] is the form of the ferric siderophore that is recognized by the FecA receptor protein. In the photoreaction of $Fe(III)_2\text{-}(cit)_2^{2-}$, both equivalents of Fe(III) are reduced per equivalent of citrate decarboxylated, consistent with the decarboxylation process being a two-electron oxidation reaction (Figure 16).[115] The 3-ketoglutarate that is formed initially is not stable in acid and further decomposes producing acetone. Ferric carboxylate complexes are also quite photoreactive, although with a reactivity somewhat less than that of ferric α-hydroxycarboxylate complexes.[115,124]

β-Hydroxyaspartate-containing siderophores: β-Hydroxyaspartic acid has also been found to be photoreactive when complexed to Fe(III). The marine siderophores that contain β-hydroxyaspartic acid include the aquachelins, loihichelins, marinobactins, alterobactins (see Figure 18, below) and pseudoalterobactins (see Figure 18, below). Photolysis of the Fe(III)-aquachelins,[101] produces a modified peptide ligand and Fe(II) (Figure 17). In fact photolysis of each aquachelin separately leads to the same oxidized peptide product, m/z 780, consistent with the loss and oxidation of the β-hydroxyaspartate amino acid and release of the fatty acid. The photoproduct coordinates Fe(III) with the two hydroxamate groups, although the complete coordination environment of this Fe(III) complex has not yet been determined. The conditional

stability constants show that the photoproduct coordinates Fe(III) with somewhat lower affinity, $10^{11.5}$ M^{-1}, than Fe(III)-aquachelin C, $10^{12.2}$ M^{-1}.[101]

The ferric complexes of the other peptide siderophores that contain β-hydroxyaspartic acid (Figure 18) are also photoreactive, although these reactions have not been investigated in detail yet. In the photolysis of the Fe(III)-loihichelins, the equivalent photoproduct to the aquachelins photoproduct is observed.[99] UV-Vis changes upon photolysis also show the loss of the UV absorption corresponding to the β-hydroxyaspartate-to-Fe(III) charge transfer band in Fe(III)-loihichelin photolysis. Similarly Fe(III)-marinobactins and Fe(III)-alterobactins display similar uv-vis changes upon photolysis. However, the ferric marinobactins, while photoreactive, produce many different products, which is most likely a combination of the radical nature to the photoreaction and the presence of the β-hydroxyamide that is coordinated to Fe(III).

3.2.3. Other marine siderophores—In addition to the marine siderophores presented in Figures 10, 14, 15, and 18, siderophores from other marine bacteria such as *Marinobacter hydrocarbonoclasticus* and *M. aquaeolei*, which produces petrobactin and petrobactin sulfonate(s), and *Aeromonas hydrophila* which produces the amonabactins, as well as fish pathogens such as *Vibrio anguillarum* which produces vanchrobactin and anguibactin are known (Figure 19). Petrobactin is a citrate siderophore, which is unique in the incorporation of 3,4-dihydroxybenzoyl (3,4-DHB) as an OO′ donor in place of the more common 2,3-dihydroxybenzoate group. Petrobactin has been isolated from *Bacillus anthracis* also (see below). However, unlike *B. anthracis*, *M. aquaeolei* produces the mono- and di-sulfonated derivatives of petrobactin, which tunes the relative hydophilicity of this series of siderophores (Figure 19). Sulfonation of the catechol group has also been observed in other marine siderophores. Pseudoalterobactin (Figure 18) has only been reported in its sulfonated form, however it is structurally related to the alterobactins (Figure 18), which have not yet been found in their sulfonated form. Thus catecholate sulfonation may be an emerging class of distinct marine siderophores.

3.3. Amphiphilic siderophores produced by pathogens and other microbes

The only other class of bacteria that has been found to produce suites of amphiphilic siderophores besides marine bacteria are certain pathogens (e.g., Mycobacteria),[129-131] although a few other bacteria have been reported to produce selected single or small groups of amphiphilic siderophores, including the ornibactins and corrugatin, peptide amphiphiles (Figure 20), as well as rhizobactin 1021 and acinetoferrin, citrate-based amphiphiles (Figure 21). The ornibactins are produced by *Burkholderia sp.* and corrugatin is produced by *Pseudomonas corrugata*.[132-134] They are rather hydrophilic acyl-appended peptide siderophores by virtue of their short fatty acid tails relative to the longer hydrophilic peptide head group.

Acinetoferrin is produced by *Acinetobacter haemolyticus*;[135] it is structurally related to schizokinen, rhizobactin 1021 and even the marine synechobactins by variation in the acyl appendage (Figure 21). While rhizobactin 1021 is thought to be synthesized from schizokinen, the biogenic precursor of the synechobactins and acinetoferrin are not yet known. Acinetoferrin, like the ornibactins lies within the hydrophobic spectrum of amphiphilic siderophores and it partitions into bilayer membranes like the other citrate siderophores (see below).[111]

Mycobacteria produce suites of two related siderophores, each containing the same head group (Figure 22). The mycobactins are lipophilic siderophores with long fatty acid tails that reside in the bacterial membrane, and the carboxymycobactins, which are released from the bacterium, are hydrophilic siderophores distinguished by shorter fatty acids that have a

carboxylic acid at the end of the fatty acid chain, such as shown in Figure 22 for mycobactin T and carboxymycobactin T produced by *Mycobacterium tuberculosis*, the causative agent of tuberculosis infections. It has been proposed that iron uptake occurs by transfer of Fe(III) from the carboxymycobactins to the membrane-associated mycobactin.[130,136] However, a new pathway for iron uptake by mycobacteria is under investigation. Mycobactin J has been shown to partition into macrophages and then to sequester Fe(III) from the macrophage iron pools. The presumably reduced membrane affinity of the Fe(III)-mycobactins would then localize them in the cytoplasm in the form of proposed self-assembled "liquid droplets".[137,138] Of importance for bacterial growth, this form of non-membrane partitioned Fe(III)-mycobactins may be more readily recognized and taken up by the mycobacterium. This new strategy suggests the mycobactins, while cell-associated, may under certain conditions be released from the bacterium and able to sequester iron from uninfected cells.

4. Microbial production of multiple siderophores to evade host defense responses

Some bacteria reportedly only secrete one type of siderophore whereas others produce multiple types of siderophores. For example, *Bacillus* species (most notably *Bacillus anthracis*, the causative agent of anthrax) produce the triscatecholamide siderophore bacillibactin as well as petrobactin (Figure 23b), the unique citrate siderophore that also utilizes two 3,4-dihydroxybenzoyl (3,4-DHB) chelating moieties.[139] While bacillibactin has a higher affinity for Fe(III) than petrobactin, the virulence of *Bacillus* species is dependent upon the production of petrobactin.[140-142] In other microbes the relationship between production of multiple siderophores and pathogenicity is not as clear. *E. coli* strains which produce only enterobactin (e.g. *E. coli* K12) are not pathogenic. Some strains of *E. coli*, *Salmonella*, and *Yersinia* which produce aerobactin, salmochelin, and yersiniabactin, in addition to enterobactin (Figure 23a) are pathogenic,[143-146] whereas others apparently are not. For example, *E. coli* Nessle 1917 which is a probiotic species, produces all four of the enterobactin, salmochelin, aerobactin and yersiniabactin siderophores.[144]

Production of multiple siderophores, however, may be advantageous for microbes to survive and grow in different environments. Yersiniabactin and salmochelin were the dominate siderophores produced by *E. coli* Nissle 1917 under neutral to alkaline conditions, whereas production of enterobactin and aerobactin increased under more acidic conditions.[144] Temperature and levels of bicarbonate appear to influence the production of siderophores in *Bacillus* species also. Petrobactin was isolated from *Bacillus* cultures grown at both 23°C and 37°C in both ambient air conditions and under conditions of a 5% CO_2 atmosphere. However bacillibactin was not detected in cultures grown at 37°C at 5% CO_2 but was detected in cultures grown at 37°C in ambient air (Figure 23b).[147] *Pseudomonas aeruginosa* also produces two siderophores pyoverdine and pyochelin (Figure 23c). Production of pyoverdine is linked to biofilm formation, a virulent trait of *P. aeruginosa* in chronic lung infections of cystic fibrosis patients,[148] and pyochelin is able to evade the mammalian immune response siderophore-binding protein, siderocalin (described below). The virulence of plant pathogen *Erwinia chrysanthemi* is dependent on the production of two siderophores: a monocatecholate siderophore, chrysobactin, as well as achromobactin, a citrate derived siderophore (Figure 23d).[89]

The production of suites of amphiphilic siderophores by marine bacteria (Figure 10) and mycobacteria (Figure 22) is a related form of production of multiple siderophores. Functionalizing a siderophore with a fatty acid increases cell membrane partitioning, as well as surface reactivity and self-assembly, but the relative amphiphilicity and extent of hydrophobicity versus hydrophilicity is also tuned. A stark example is the production of the fully hydrophilic form of the head group, as in aerobactin (Figure 9), and the hydrophobic

ochrobactins A-C (Figure 10), which are amphiphilic forms of aerobactin, by the marine bacterium, *Ochrobactrum sp.* SP18.[96] Adapting to a change of hydrophilicity in the surrounding environment may be essential to bacterial survival.

Production of multiple siderophores is also advantageous for infectious pathogenic bacteria striving to sequester iron from the host's tightly secured iron stock. In response to infection, some mammalian hosts have been found to produce a lipocalin-type protein, siderocalin (also known as lipocalin 2, neutrophil gelatinase-associated lipocalin (NGAL), 24p3, uterocalin, or *neu*-related lipocalin), which binds select apo and ferric siderophore complexes with high affinity[80,155-158] (e.g ferric enterobactin $K_D = 0.4 \pm 0.04$ μM). The x-ray structure reveals that the binding site of siderocalin is shallow and lined with positive residues (K125, K134, and R81), which is unusual compared to other lipocalins, allowing for siderocalin to bind several different siderophores (Figure 24).[159,160]

Siderocalin has been shown to irreversibly bind ferric complexes of catecholate siderophores utilizing 2,3 dihydroxybenzoic acid (DHBA) such as enterobactin, bacillibactin, 2,3 DHBA as well as the carboxymycobactins, the soluble siderophores produced by mycobacteria, thus preventing bacterial iron acquisition via production of these siderophores.[80,158] However, bacteria have overcome this problem by producing more than one siderophore, and while one of the siderophores may be sequestered by siderocalin, the other “stealth” siderophore is not recognized by the protein.[140] Such stealth siderophores include pyochelin, aerobactin, petrobactin and salmochelins, which are not bound by siderocalins. In addition the tris-hydroxamate siderophores, which form neutral ferric complexes, are also not bound by siderocalin.[140,161] Aerobactin and petrobactin have much lower affinities for iron(III) than enterobactin and bacillibactin, however their production is essential for bacterial virulence.

5. Summary and challenges for the future

As a result of the importance of iron for growth, bacteria have evolved multiple parallel pathways for iron uptake (Figure 1). In this review an overview of the iron acquisition strategies bacteria use to acquire iron was presented, with a specific focus on siderophore structures, particularly those produced by oceanic bacteria. While the siderophores themselves are only one component of the overall iron acquisition process, relatively little is known about the other components of siderophore-mediated iron acquisition in oceanic bacteria, including membrane receptor-mediated transport pathways, regulatory mechanisms, as well other routes for iron uptake, through, for example, FeHeme acquisition or from the transferrin-type proteins or ferric reductase pathways. A notable exception is the fish pathogen *V. anguillarum*.[162-164]

Given the paucity of iron in ocean water, the marine environment presents challenges to microorganisms in their quest to obtain the iron that is required for growth. Marine bacteria have responded by producing suites of amphiphilic siderophores, many of which are photoreactive when coordinated to Fe(III). While these features set marine siderophores apart from the majority of siderophores produced by terrestrial microbes, elucidating the biological advantages conferred by these structural traits requires further studies. Some pathogenic bacteria, which must also survive and thrive in very low iron environments, have also been found to produce amphiphilic siderophores. Thus investigations into the effects of amphiphilicity on surface activity and particle interactions (e.g., iron oxides), and in membrane partitioning, as well as the role that self-assembly might play in the iron acquisition process are needed. UV light has been shown to affect iron acquisition mediated by photoreactive Fe (III)-siderophore complexes in a source bacterium[100,101,120] as well as in other organisms in culture with the source bacterium[101,120], yet we know relatively little about recognition and uptake by the other organisms.

Iron uptake by heme acquisition is a preferred strategy by pathogenic microbes, and some marine pathogens such as *V. anguillarum* have a well defined heme uptake system.[164] But recently oceanic bacteria have been shown to have a heme-mediated iron uptake system also.[165] *Microscilla marina* reportedly grows on heme as its sole iron source. The genome of *M. marina* reveals a cluster of genes with similarity to known heme uptake genes (e.g., heme receptor, heme oxygenase, etc) that are upregulated when *M. marina* is grown on heme. Moreover analysis of about 150 marine bacterial genomes suggests that many marine bacteria may be able to use a heme transport pathway to acquire iron. Genomics will undoubtedly continue to play a pivotal role in further elucidation of iron uptake pathways. With the widely stated estimate that less than one percent of the planet's microbes have been brought into culture, the challenge now includes development of new methods to bring more microbes into culture, as well as to identify other possible iron uptake pathways in a wider range of microbes.

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Biographies

Alison Butler obtained a B.A. in Chemistry from Reed College in 1977 and a PhD in inorganic chemistry from the University of California San Diego in 1982. After an NIH Postdoctoral fellowship at UCLA and Caltech, she joined the faculty at UC Santa Barbara in 1986, where she is a Professor of Chemistry and Biochemistry. Her research interests are in bioinorganic chemistry and metallobiochemistry, including mechanisms of metal acquisition by microbes and mechanistic chemistry of metalloenzymes.

Moriah Sandy is a native of San Diego, California. She received her B.S. with honors in chemistry from the University of Redlands in 2005, where she did her undergraduate research with Prof. David Soulsby. As an undergraduate, she was also a research intern for a summer at The Scripps Research Institute in La Jolla, CA, in the laboratory of Prof. Ashok Deniz (2004). Moriah is currently a Ph.D candidate in the Department of Chemistry and Biochemistry at the University of California, Santa Barbara, under the direction of Prof. Alison Butler. She is a recipient of a National Science Foundation East Asia and Pacific Summer Institute Award (2007-8) where she worked for a summer with Prof. John Blunt and Prof. Murray Munro at the University of Canterbury, New Zealand, and she recently received a UCSB Roche Bioscience Graduate Excellence Award (2009). Her general research interests are in bioinorganic chemistry, metallobiochemistry, and natural products chemistry.

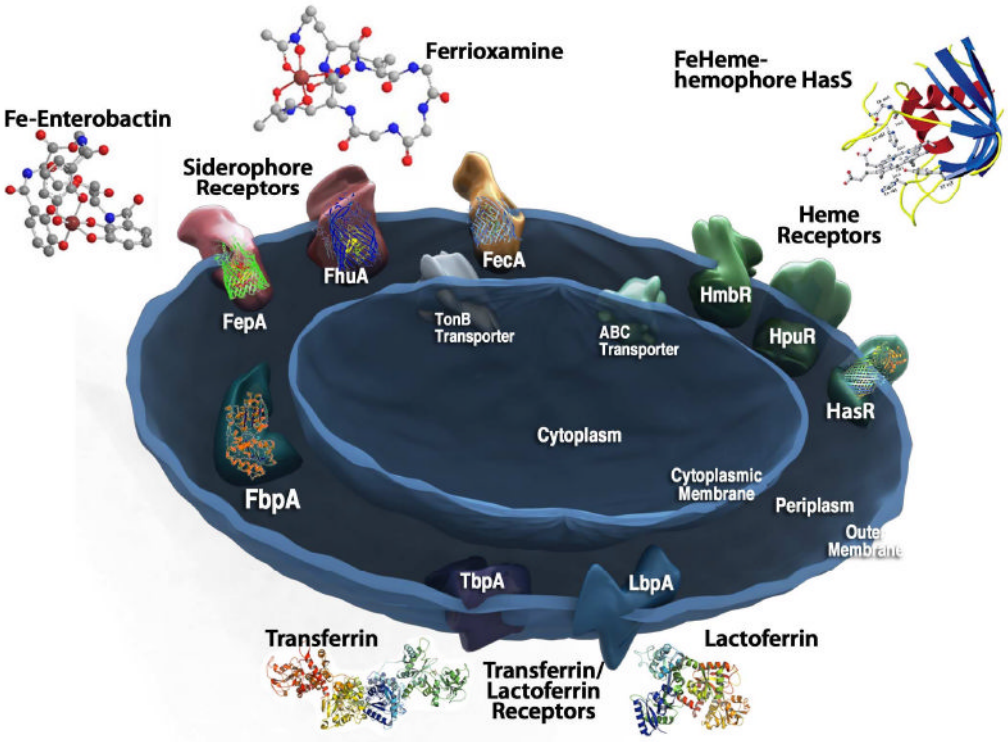

Figure 1.
Microbial (Gram negative) iron uptake pathways.

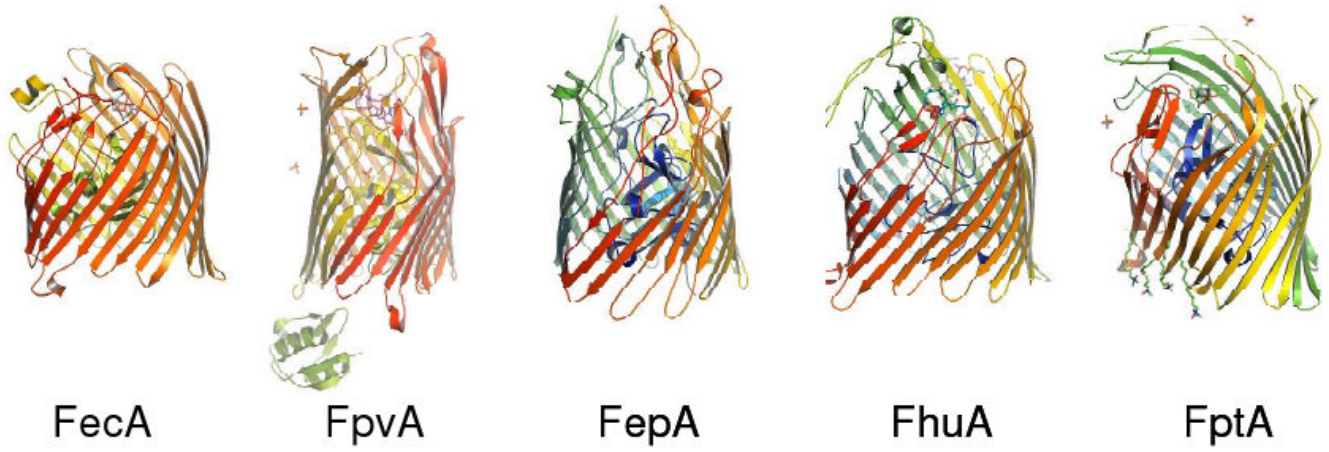

Figure 2.
Ribbon diagrams of outer membrane siderophore receptor proteins from *E. coli*: ferric-citrate (FecA), ferric-enterobactin (FepA) and ferric-hydroxamate (FhuA); and *P. aeruginosa*: ferric pyoverdine (FpvA) and ferric pyochelin (FptA).

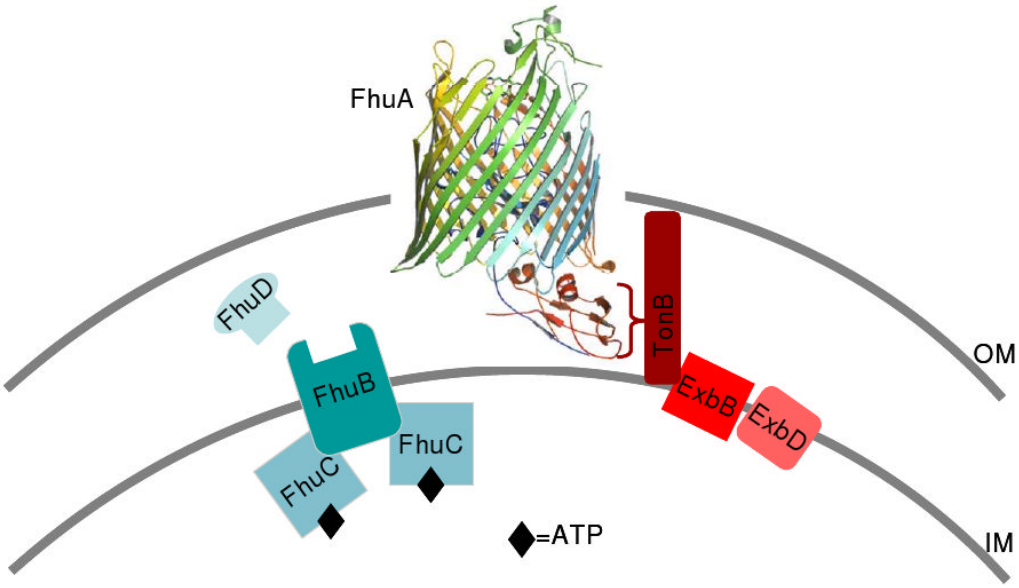

Figure 3.
Schematic of the proteins involved in ferrichrome transport. The crystal structure of FhuA in complex with the C-terminus of TonB was reported by Pawelek et al, 2006.[28]

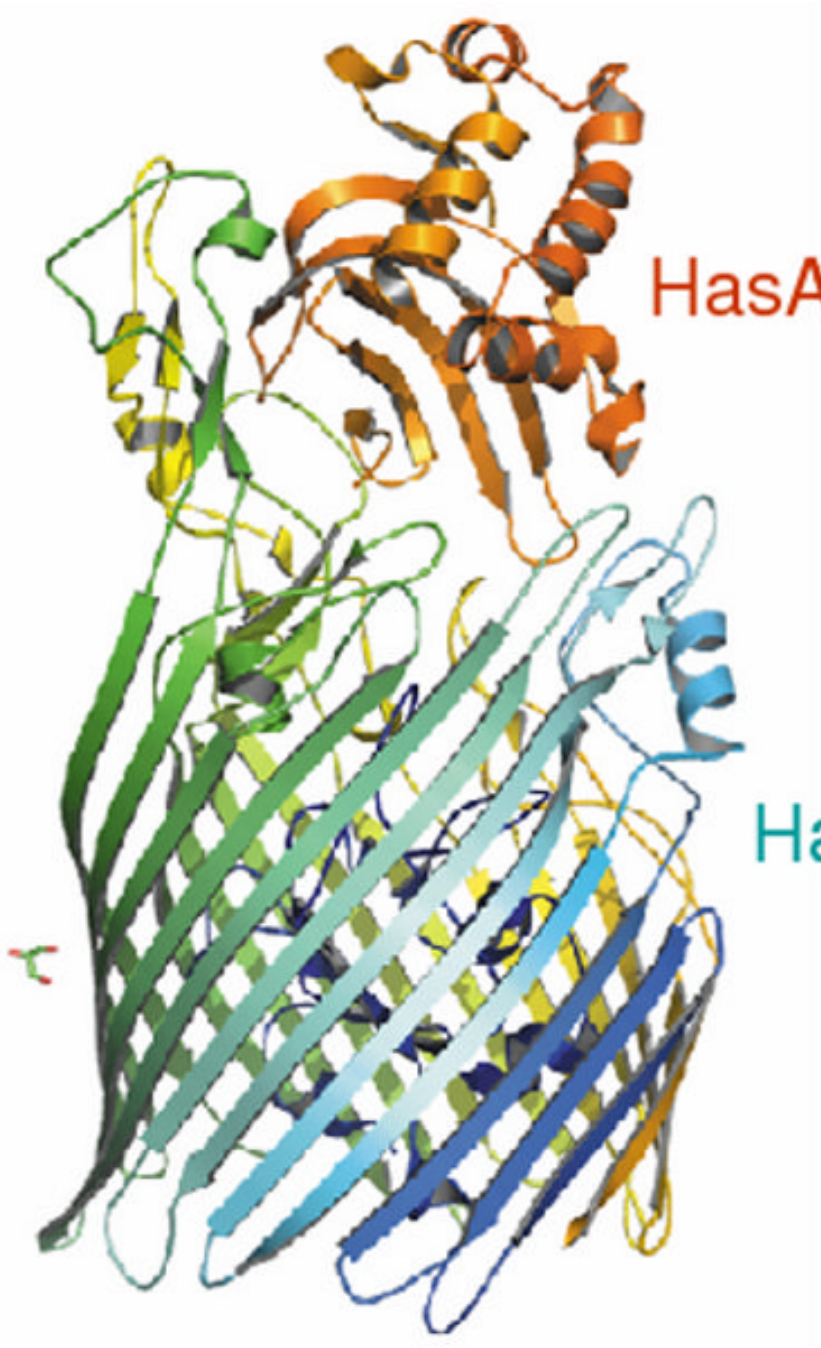

Figure 4.
Ribbon representation of the *S. marcescens* hemophore, HasA (red), bound to its outer membrane receptor protein HasR (blue) (PDB code 3CSN).[33]

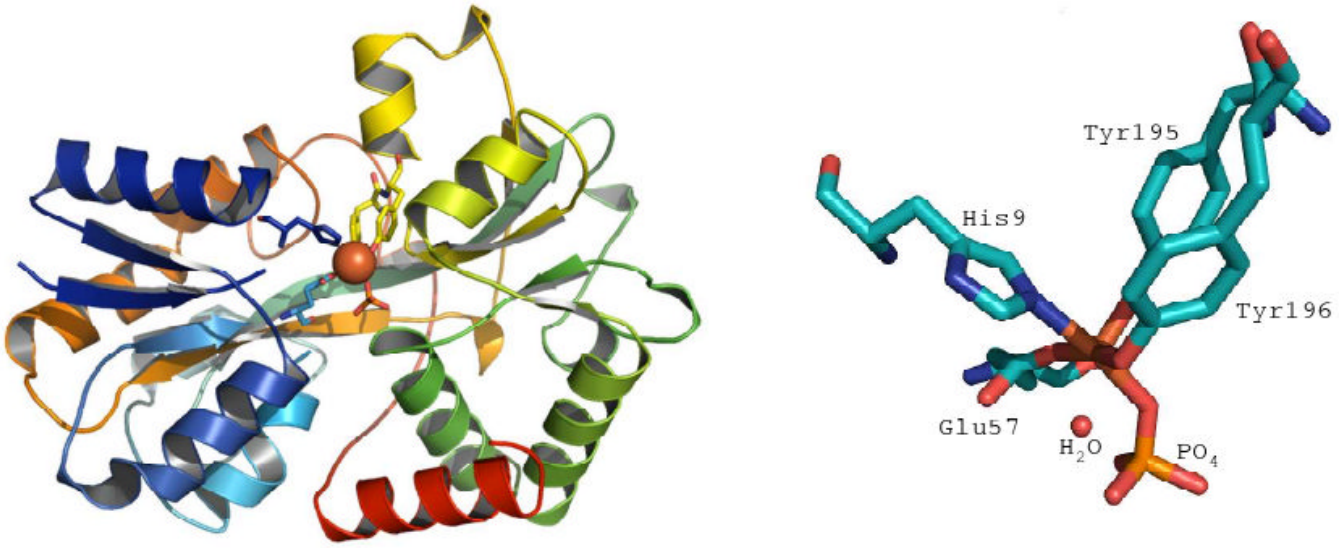

Figure 5.
Ribbon diagram depiction of the *Haemophilus influenza* Fbp protein, the ferric binding site is shown on the right. The ferric ion is coordinated by two oxygens from Tyr195 and Tyr196, an imidazole nitrogen from His9, a carboxylate oxygen from Glu57, an oxygen atom from an exogeneous phosphate anion, and an oxygen atom from a water molecule in an octahedral arrangement. (PDB Code 1MRB)[47]

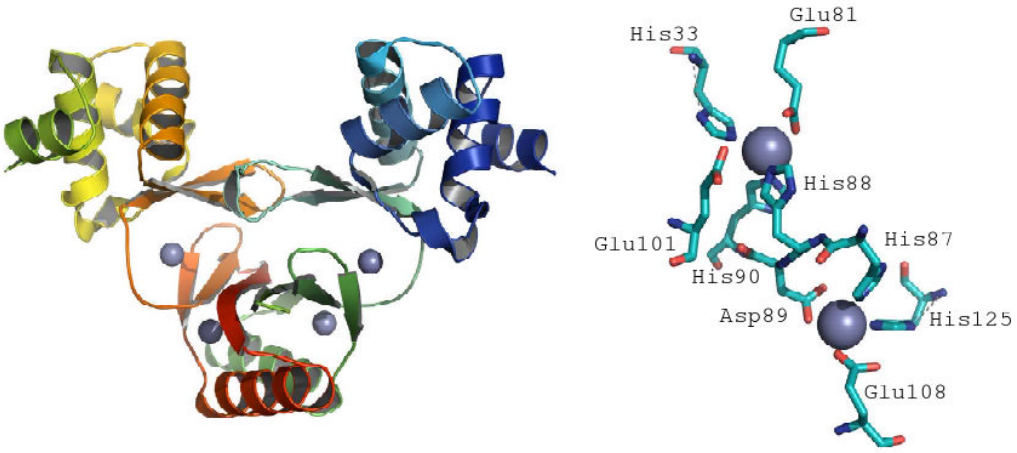

Figure 6.
Crystal structure of the ferric uptake regulator (Fur) protein from *Vibrio cholerae*.[59]

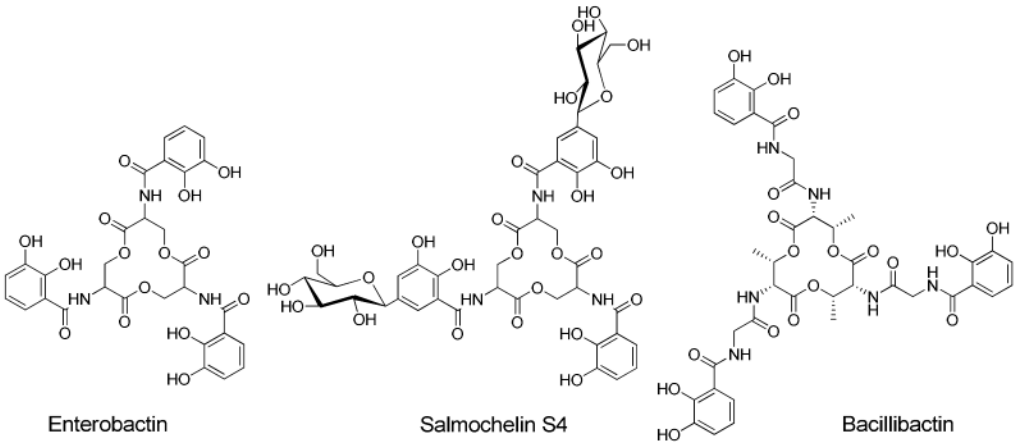

Figure 7.
Structures of enterobactin, salmochelin S4 and bacillibactin.

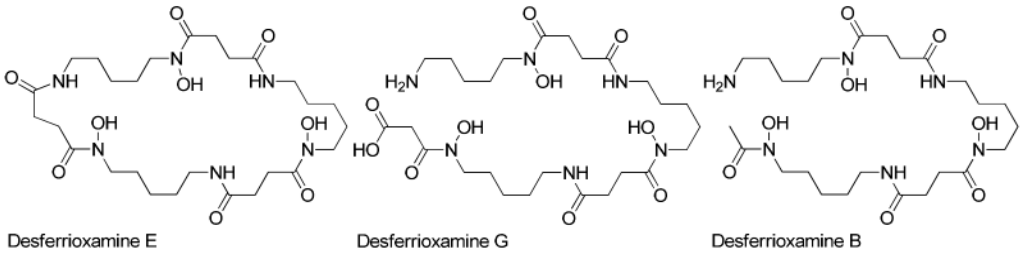

Figure 8.
Structures of desferrioxamines E, G and B.

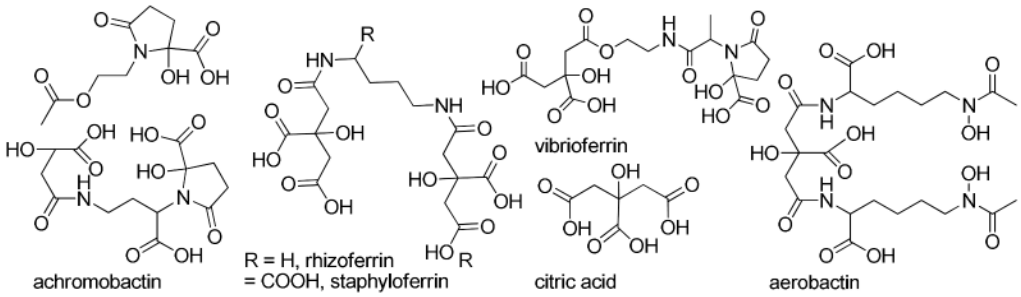

Figure 9.
Structures of selected α-hydroxycarboxylate siderophores.

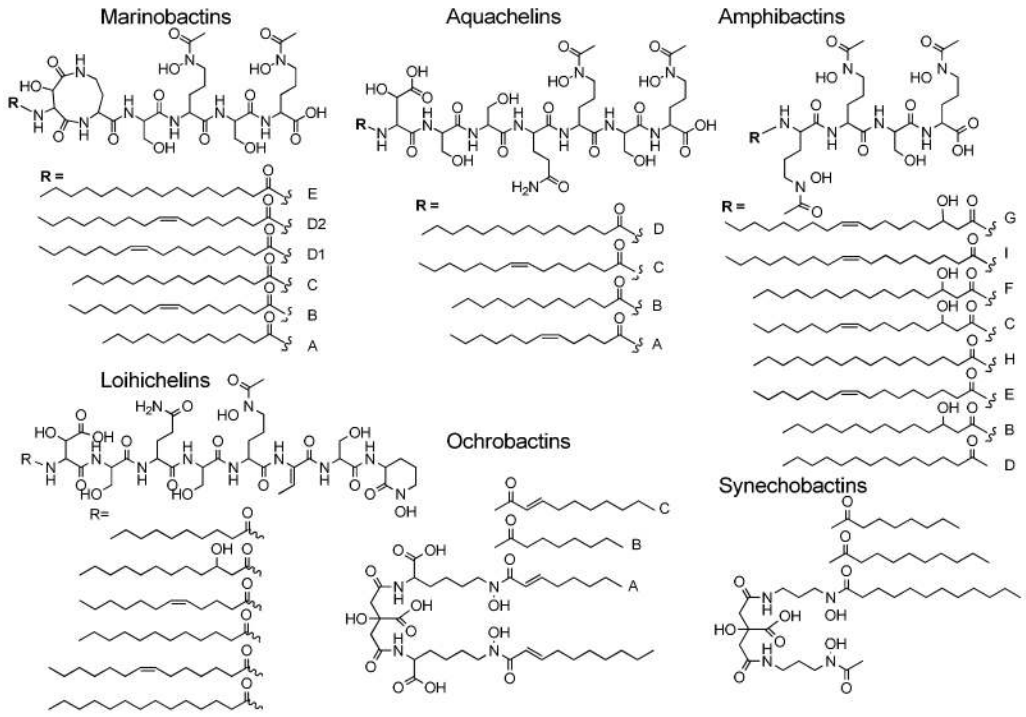

Figure 10.

Suites of marine amphiphilic siderophores: marinobactins (*Marinobacter sp.* DS40M6) [106-110] and aquachelins (*Halomonas aquamarina* DS40M3);[98] amphibactins (*Vibrio* sp. R10); [97] loihichelins (*Halomonas* sp. LOB-5);[99] ochrobactins (*Ochrobactrum* sp. SP18);[96] synechobactins (*Synechococcus* sp. PCC 7002).[95]

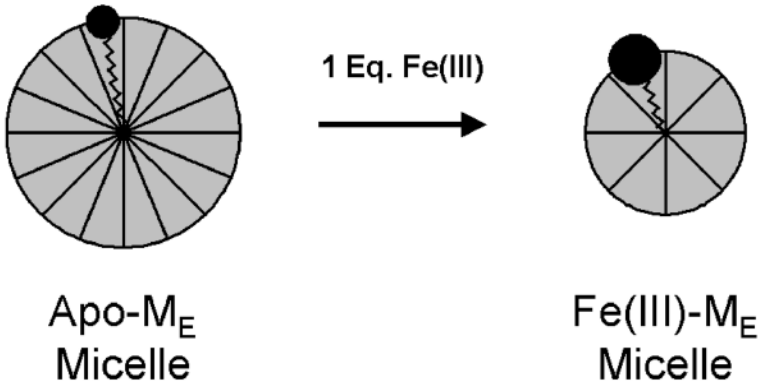

Figure 11.
Coordination of Fe(III) could give M_E a larger head group area : tail volume ratio such that a smaller micelle is formed.[107] Reproduced from [reference 107].

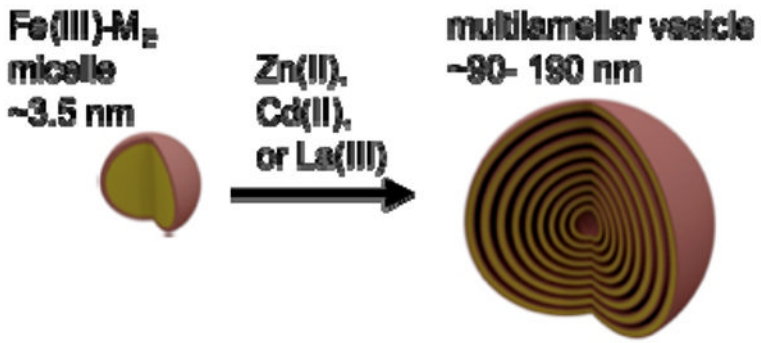

Figure 12.
Multilamellar vesicle formation from Fe(III)-marinobactin E induced by addition of Zn(II), Cd(II), La(III) or excess Fe(III). Adapted from reference. [109]

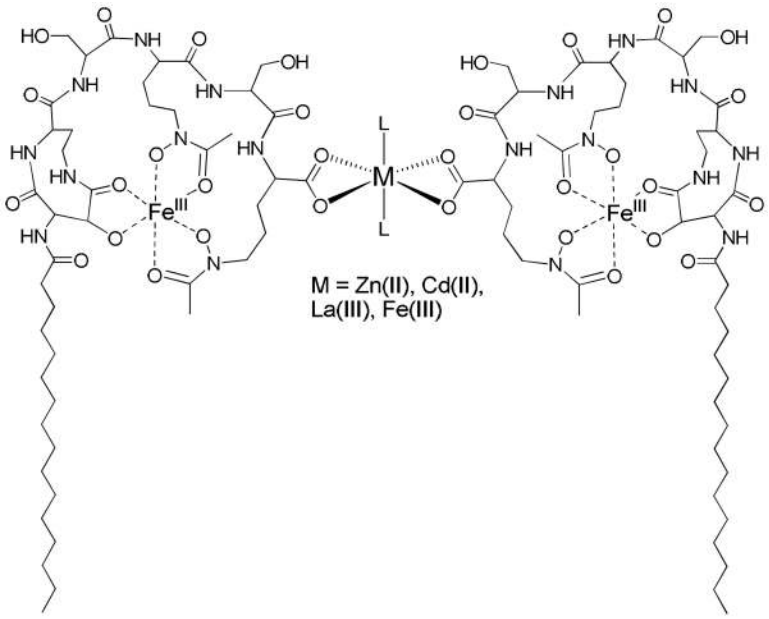

Figure 13.

Proposed terminal carboxylate crosslinking of marinobactin E by the added cations, M (Zn(II), Cd(II), La(III) or excess Fe(III)). The bis-bidentate coordination geometry of the two carboxylates shown in the figure could also be bis-monodentate carboxylate cross linking. The resulting "composite surfactant" would have a lower headgroup-area : tail-volume ratio that may favor vesicle formation. "L" is an undefined ligand to fill out the octahedral coordination.[112]

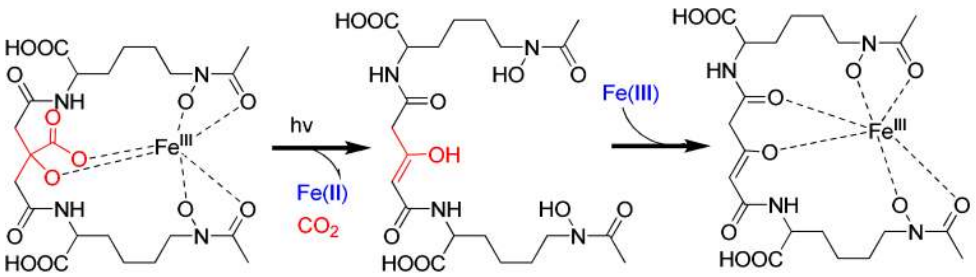

Figure 14.
Reaction scheme for the uv photolysis of Fe(III)-aerobactin under aerobic conditions.[100]

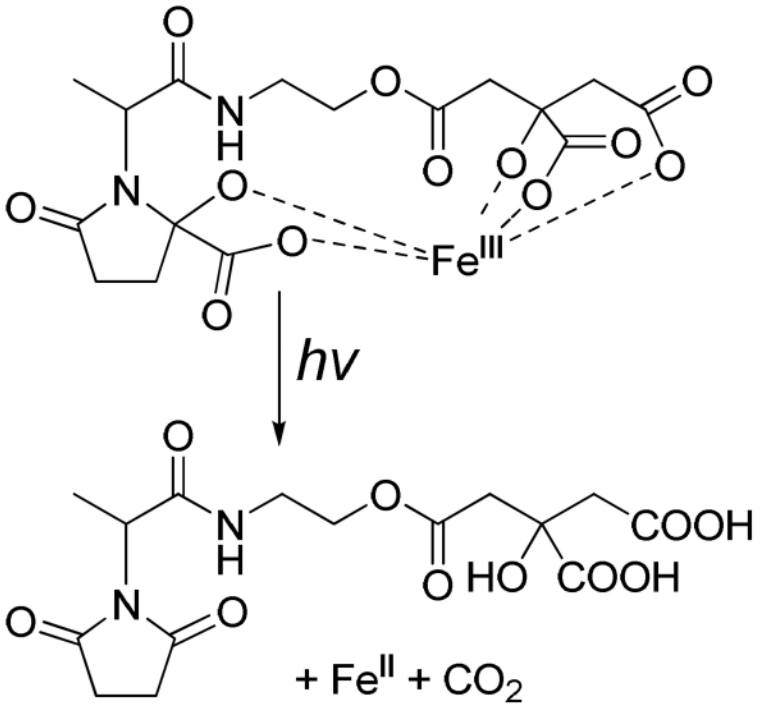

Figure 15.
Photoreaction of Fe(III)-vibrioferrin. Reaction derived from data presented in [reference 120].

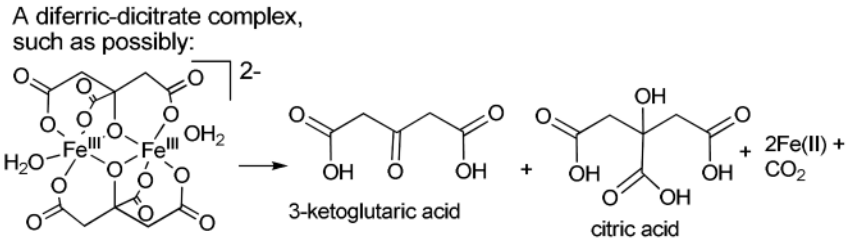

Figure 16.

Proposed photoreaction of diferric dicitrate in acid. Reaction derived from data presented in reference 114.

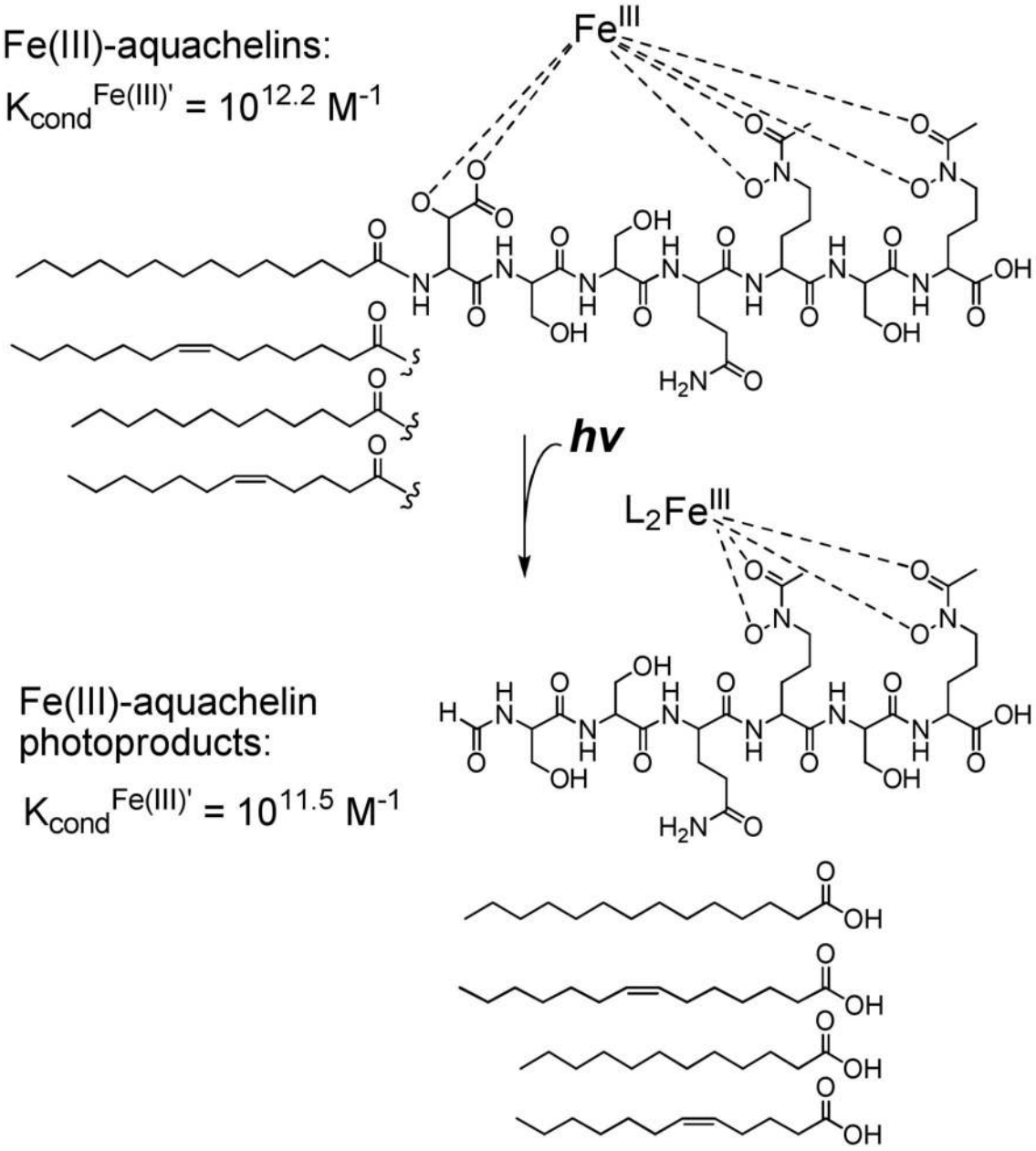

Figure 17.
Photoreaction of Fe(III)-aquachelin. "L" is an undefined ligand to fill out the octahedral coordination.[101]

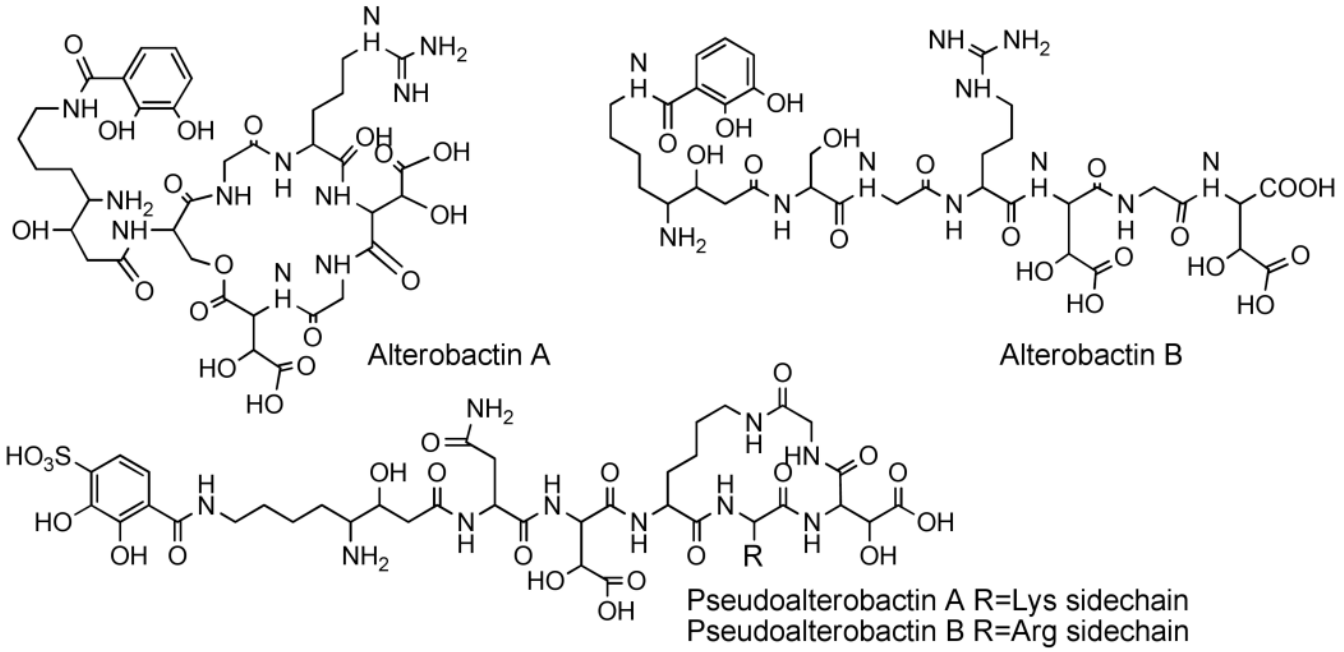

Figure 18.
Structures of other marine peptide siderophores that contain β-hydroxyaspartic acid.

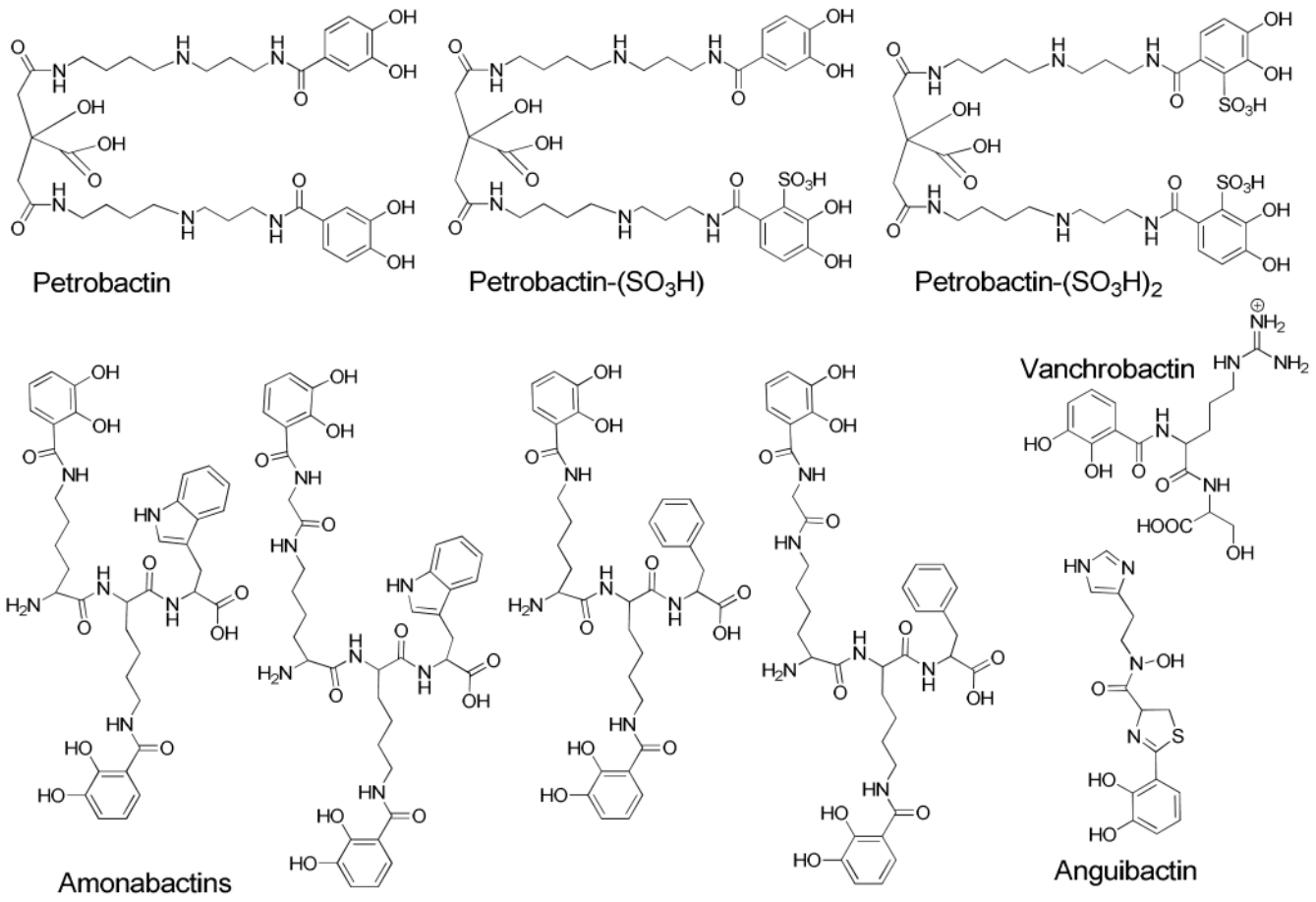

Figure 19.

Other siderophores produced by marine pathogens and oceanic bacteria: petrobactin, petrobactin-(SO_3H), and petrobactin-(SO_3H)$_2$ (*M hydrocarbonoclasticus, Marinobacter aquaeolei VT8*);[103,104,125] vanchrobactin and anguibactin (*Vibrio anguillarum*);[126,127] amonabactins (*Aeromonas hydrophila*).[128]

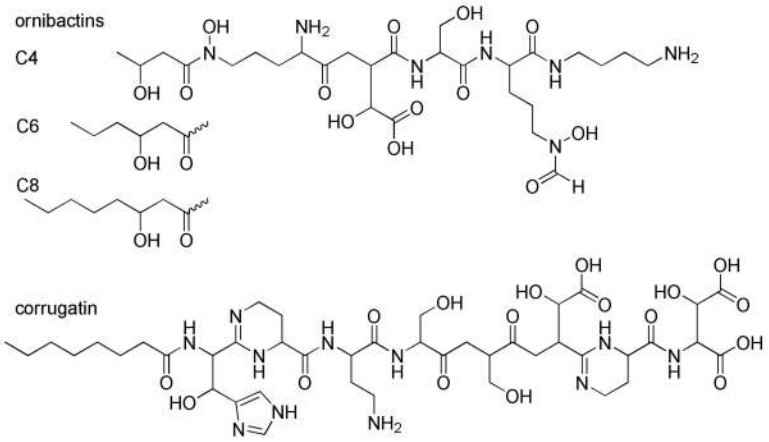

Figure 20.
Structures of the ornibactins and corrugatin.

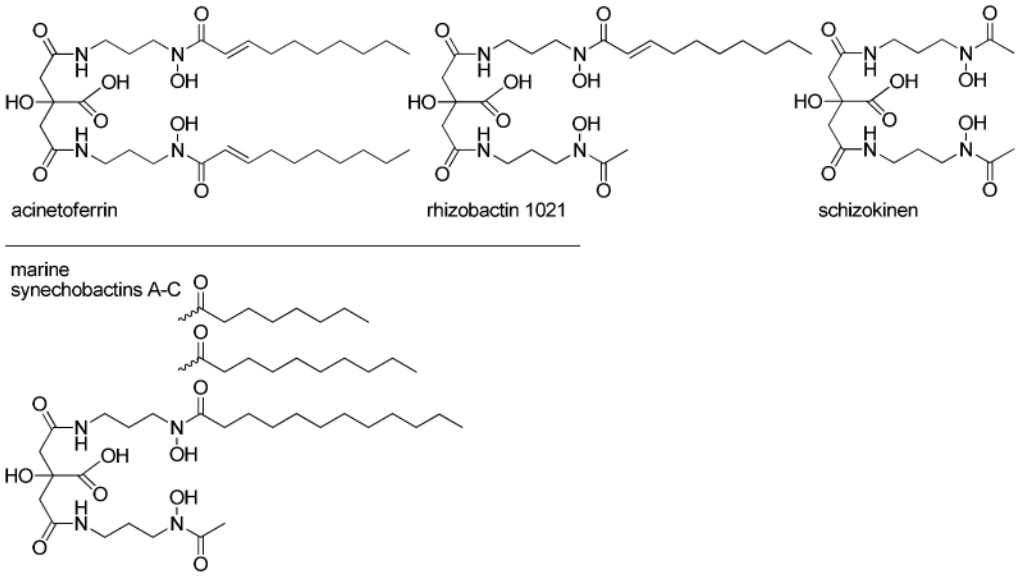

Figure 21.
Comparison of the amphiphilic citrate siderophores of acinetoferrin rhizobactin 1021 and the synechobactins to the hydrophilic schizokinen siderophore.

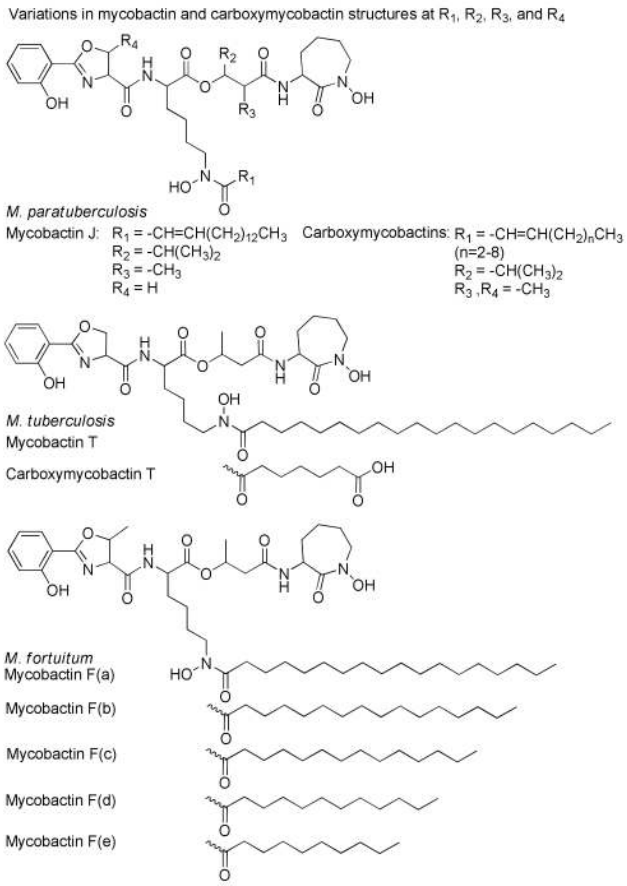

Figure 22.
Structures of mycobactins and carboxymycobactins produced by *Mycobacteria*.

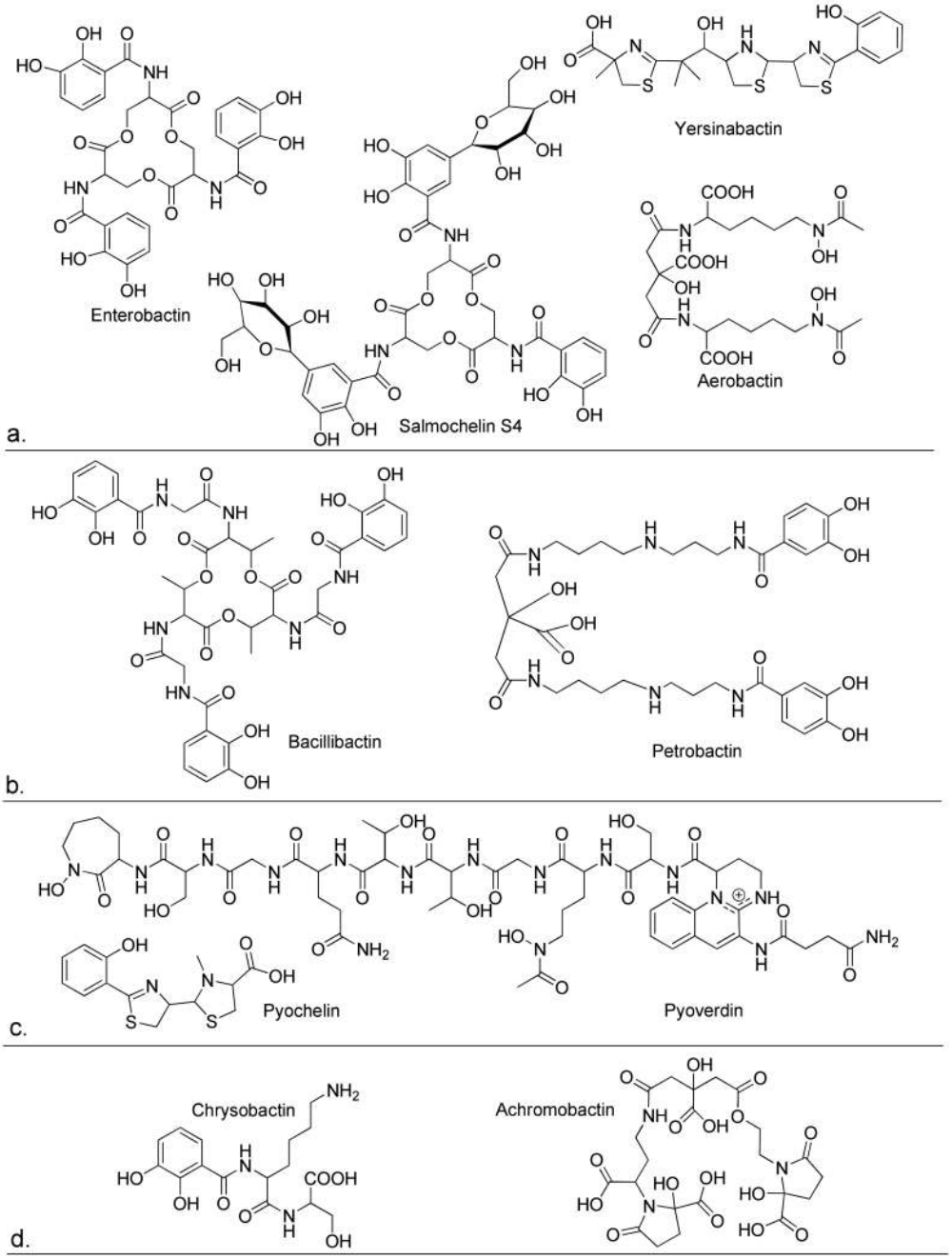

Figure 23.

Multiple siderophores produced by different pathogenic bacteria: enterobactin,[149,150] salmochelins,[75] aerobactin,[116,151] and yersinabactin[152] (*E. coli*, *Salmonella*, and *Yersinia* sp.); bacillibactin and petrobactin (*Bacillus* sp.);[139] pyochelin and pyoverdin *(P. aeruginosa)*; chrysobactin and achromobactin (*E. chrysanthemi*).[153,154]

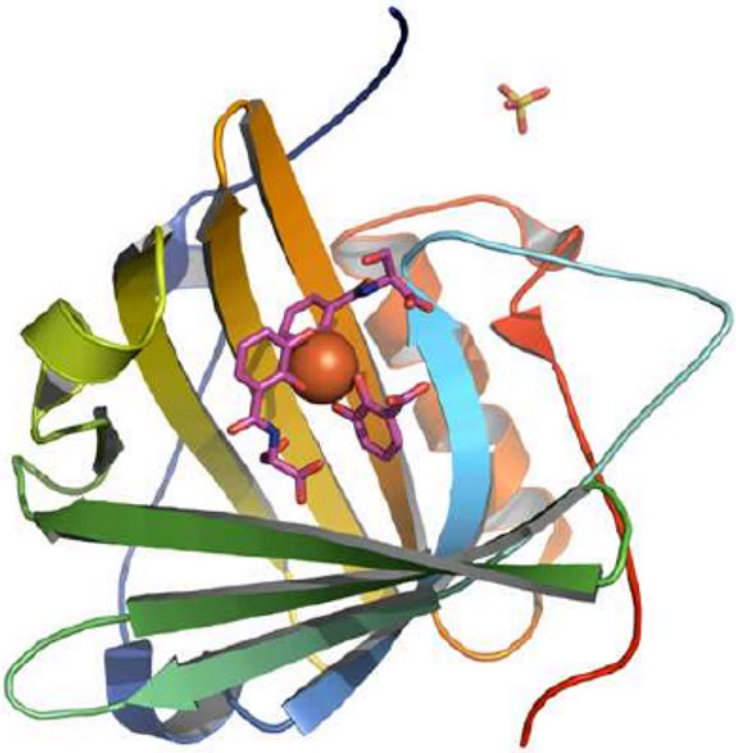

Figure 24.
Ribbon representation of siderocalin bound to ferric enterobactin (PDB code 3BYO).[159]