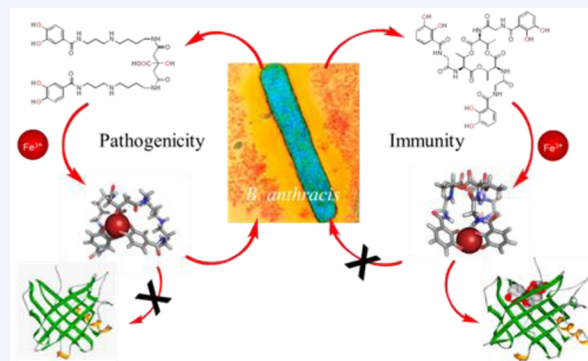


Coordination Chemistry of Microbial Iron Transport

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CONSPECTUS: This Account focuses on the coordination chemistry of the microbial iron chelators called siderophores. The initial research (early 1970s) focused on simple analogs of siderophores, which included hydroxamate, catecholate, or hydroxycarboxylate ligands. The subsequent work increasingly focused on the transport of siderophores and their microbial iron transport. Since these are pseudo-octahedral complexes often composed of bidentate ligands, there is chirality at the metal center that in principle is independent of the ligand chirality. It has been shown in many cases that chiral recognition of the complex occurs. Many techniques have been used to elucidate the iron uptake processes in both Gram-positive (single membrane) and Gram-negative (double membrane) bacteria. These have included the use of radioactive labels (of ligand, metal, or both), kinetically inert metal complexes, and Mössbauer spectroscopy. In general, siderophore recognition and transport involves receptors that recognize the metal chelate portion of the iron–siderophore complex. A second, to date less commonly found, mechanism called the siderophore shuttle involves the receptor binding an apo-siderophore.



Since one of the primary ways that microbes compete with each other for iron stores is the strength of their competing siderophore complexes, it became important early on to characterize the solution thermodynamics of these species. Since the acidity of siderophores varies significantly, just the stability constant does not give a direct measure of the relative competitive strength of binding. For this reason, the pM value is compared. The pM , like pH , is a measure of the negative log of the free metal ion concentration, typically calculated at pH 7.4, and standard total concentrations of metal and ligand. The characterization of the electronic structure of ferric siderophores has done much to help explain the high stability of these complexes.

A new chapter in siderophore science has emerged with the characterization of what are now called siderocalins. Initially found as a protein of the human innate immune system, these proteins bind both ferric and apo-siderophores to inactivate the siderophore transport system and hence deny iron to an invading pathogenic microbe. Siderocalins also can play a role in iron transport of the host, particularly in the early stages of fetal development. Finally, it is speculated that the molecular targets of siderocalins in different species differ based on the siderophore structures of the most important bacterial pathogens of those species.

INTRODUCTION

In 1970, Professor John “Joe” Neilands of the (then) Berkeley Biochemistry Department gave a seminar in the Inorganic Chemistry series regarding newly characterized microbial iron chelators. As a new member of the Berkeley faculty, the senior author had been assigned as chair of this seminar series and issued the invitation. At the time, this was very unconventional, since biochemists did not routinely speak at such a venue. However, this was at the beginning of a movement that was to become “bioinorganic chemistry”, an oxymoron that has endured as the descriptor of a field that has developed into a major and vigorous scientific discipline. Neilands, along with Walter Keller-Schierlein of the ETH in Zurich were early pioneers in the field who elucidated the chemical structures and function of what became known as siderophores (Greek: $\sigma\iota\delta\eta\rho\omicron\varsigma$ (iron) + $\varphi\epsilon\rho\omega$ (carry) = *iron carrier*). In his seminar, Neilands described the crystal structure of ferric ferrichrome, determined by Templeton and Zalkin at Berkeley,¹ and the role it played in iron transport through a recognition process that

occurred at receptor proteins embedded in the microbial outer membrane. When asked whether the chirality at the metal center (Δ or Λ , a chirality in principle separate from the peptide chirality) made a difference to protein recognition, Neilands replied that, as far as he knew, no one had thought about this. This question inspired the start of what became a 42 year project to explore the coordination chemistry and biological iron transport role of siderophores. For the first 10 years of this endeavor the senior author was the only chemist studying siderophores. As a junior faculty member at the time, this foray into a new field was somewhat daunting, but today siderophores and related iron transport topics are important components of medicine, chemical biology, and related disciplines.

The first progress on this project was reviewed in an Account in 1979 that continues to have approximately 300 citations each

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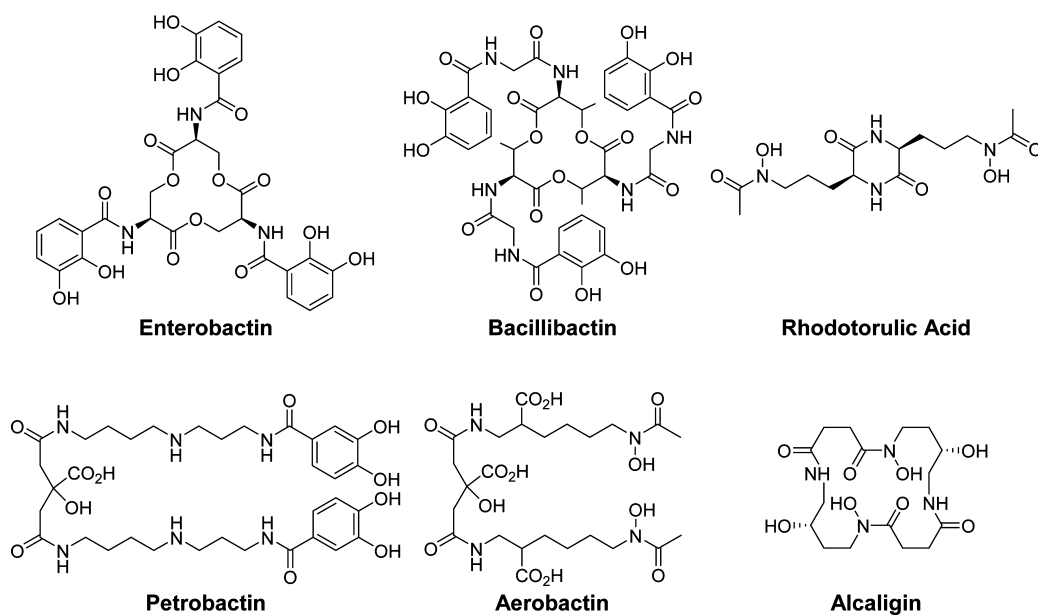


Figure 1. Chemical structure of siderophores with various iron-binding moieties; including catecholates (enterobactin, bacillibactin, petrobactin), hydroxamates (rhodotorulic acid, aerobactin, alcaligin), and carboxylates (petrobactin, aerobactin).

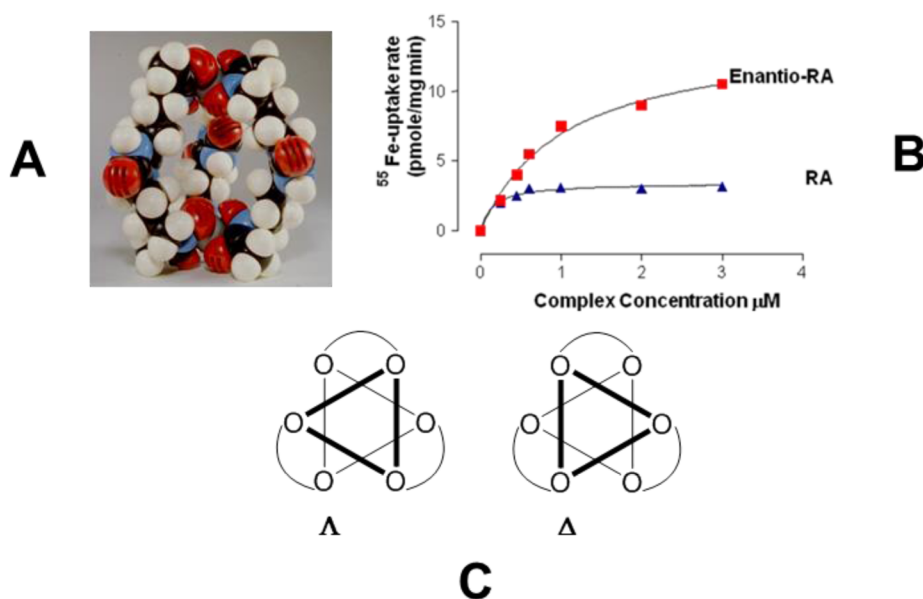


Figure 2. Chirality of metal–siderophore complex. (A) Rhodotorulic acid forms a complex with two metals and three siderophores. (B) The *E. coli* ferrichrome receptor FhuE transports native rhodotorulic acid (Δ configuration) less than the unnatural enantiomer (Λ configuration). (C) Propeller chirality of Λ (left) and Δ (right) configurations.

year.² This Account is intended to be a closing bookend to that opening one. The properties of siderophores, their iron transport functions, the dramatic expansion of knowledge about receptors, and the discovery of siderocalins in the human immune system have all been described in major reviews by us^{3–7} and others^{8,9} and will not be repeated here. The purpose of this Account is to provide a description of the current status of the field, from a rather personal perspective, and enough examples to support the narrative.

The study of siderophores goes back to 1912, when Twort and Ingram reported the discovery of mycobactin,¹⁰ the first compound that today would be called a siderophore. The mycobactin–aluminum complex was crystallized in 1949. But even then, it was not until the mid-1950s that mycobactin,

ferrioxamine B, and related compounds were identified as iron transport agents.¹¹ Walter Keller-Schierlein (on the faculty at the ETH from 1947 to 1987) first reported the trihydroxamate compound ferrioxamine.¹² Meanwhile, in the United States, Neilands discovered ferrichrome in 1952¹³ and proposed an iron transport role for it in 1957.¹⁴ These authors, their students, and others who entered the field discovered a new and important aspect of microbial biology, as described in reviews referenced herein. The siderophores were initially called siderochromes due to the intense color of these complexes.¹⁵ The red color is due to ligand to metal charge transfer, which was later spectroscopically assigned¹⁶ and the bonding further analyzed and explained.¹⁷ Since it is the iron transport function of these agents that is their significance, the

name siderophore was proposed, and this has remained the standard nomenclature for the field.¹⁸

■ SIDEROPHORE CHIRALITY

Like ferrioxamine,¹² ferrichrome is a trihydroxamate siderophore, and its chiral scaffold makes the iron complex chiral at the metal center. This feature of the natural compound raised the question of how chirality at the metal center played a role in microbial recognition and transport. Our studies of this relationship stimulated our early contributions to the siderophore field.¹⁹ Substantially later, an effect of metal center chirality on recognition by microbial protein receptors was demonstrated for the first time with the siderophore rhodotorulic acid and the ferrichrome uptake system of *Escherichia coli*, which is specific for the Λ chirality at the metal center (Figures 1 and 2).²⁰

We later showed how the chirality of the metal center of the triscatechol siderophore enterobactin influences siderophore uptake, transport, and release of iron from the siderophore to be utilized by the bacteria. The triserine backbone of enterobactin is chiral, and metal–enterobactin complexes in solution and in the solid state adopt the Δ configuration (Figure 2). Enterobactin supports growth of *E. coli* K12, but enantio-enterobactin, which forms the Λ configuration, does not. It was initially assumed that this was because recognition by the outer membrane receptor depends on the metal center and that the Δ configuration is preferred over the Λ of the unnatural enantiomer. However, it was later shown that the outer membrane receptor FepA binds enantio-enterobactin with similar affinity as enterobactin. Another protein involved in the transport of enterobactin, the periplasmic binding protein FepB, also binds both enterobactin and enantio-enterobactin with high affinity. Thus, the delivery of iron solely by the natural enterobactin stereoisomer is not due to selectivity at the outer membrane or the periplasm but occurs at a later stage²¹ by the Fes esterase (Figure 3).²² This protein is 45 608 Da (400 residues) and has two domains connected by a 20 amino acid loop. The C terminal domain carries out the catalytic cleavage of either enterobactin or its iron complex. The N terminal domain has a high sequence homology with

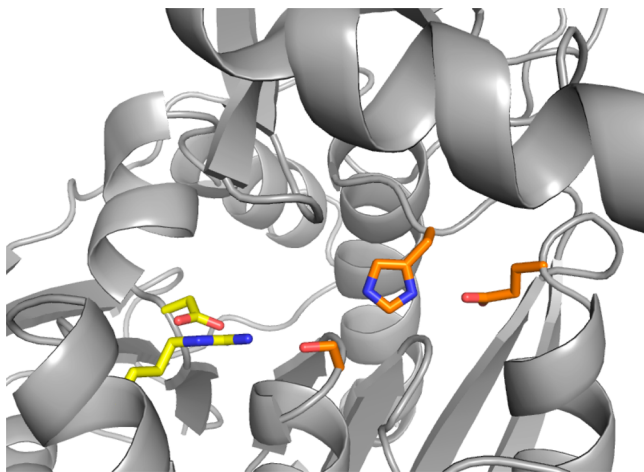


Figure 3. Active site of the enterobactin hydrolase Fes. The crystal structure of Fes from *Shigella flexneri* (PDB entry 2B20) shows an active site buried deep within the enzyme scaffold (gray ribbon). The active site is composed of a putative oxyanion hole (yellow sticks) and catalytic (orange sticks) residues.²³

ferric enterobactin (FeEnt) binding proteins. The enzyme does not accept enantio-enterobactin or its iron complex as a substrate.

To understand the role of chirality in iron delivery by enterobactin, a natural siderophore from *Bacillus subtilis* and its delivery pathway became important. Bacillibactin is structurally similar to enterobactin (Figure 1), but the metal complexes of bacillibactin preferentially form the Λ configuration. The opposite chirality results from the methylated trilactone ring made from *L*-threonine and the glycine spacer between the backbone and catechol amides. The Λ configuration is predicted by density functional theory to be lower in energy than the nearest Δ isomer by 3.6 kcal/mol. A synthetic derivative of bacillibactin that has an unmethylated *L*-serine trilactone backbone has a smaller predicted preference for the Λ isomer of 1.5 kcal/mol. Remarkably, the natural bacillibactin Λ iron complex can be converted to the Δ when bound by FeuA from *B. subtilis*, which recognizes only the Δ handedness.²⁴

The configuration of ferric enterobactin and bacillibactin was shown to be important in the iron release mechanism as just explained. Because of the strong ferric ion binding, and hence low reduction potential, these siderophores must be hydrolyzed to facilitate iron release. The enzymes Fes in *E. coli* and BesA in *B. subtilis* hydrolyze ferric enterobactin and ferric bacillibactin, respectively. Fes can hydrolyze ferric enterobactin (Δ) and *L*-serine bacillibactin (Λ) suggesting that the chirality of the backbone, not the metal center, is a prerequisite for this process. BesA can hydrolyze all siderophores that use an *L*-serine or *L*-threonine trilactone, independent of the metal center configuration, including enterobactin, bacillibactin, and *L*-serine bacillibactin. Neither BesA nor Fes can hydrolyze catecholate siderophore analogs that have a *D*-serine lactone. The chiral recognition of Fes explains why enantio-enterobactin did not deliver iron to support growth of *E. coli* even though the receptor and periplasmic binding protein recognize it. Both metal binding and siderophore chirality influence the conformation and configuration of the ferric siderophore complexes, and these shape features determine protein–siderophore interactions with significant biological consequences.

■ SIDEROPHORE UPTAKE

In 1986, we demonstrated the use of Mössbauer spectroscopy to monitor the mechanism of ⁵⁷Fe-enterobactin (⁵⁷Fe-Ent) uptake in live *E. coli*.²⁵ This was the first example of using the Mössbauer technique to investigate the oxidation state and coordination environment of ⁵⁷Fe (a Mössbauer active nucleus) during transport from extracellular space to the cytoplasm. The outer membrane protein of *E. coli*, FepA, delivers both ⁵⁷Fe-Ent and ⁵⁷Fe-MECAM (1,3,5-*N,N',N''*-tris(2,3-dihydroxybenzoyl)-triaminomethylbenzene), a synthetic enterobactin analog with a nonhydrolyzable backbone, to the periplasmic space of the cell. However, iron imported into the cytoplasm by each of these complexes occurs at different rates. The amount of ⁵⁷Fe(II) measured in the cytoplasm after 30 min of metabolism was greater when the starting complex was ⁵⁷Fe-Ent. The rate of cytoplasmic internalization of ⁵⁷Fe(II) from ⁵⁷Fe-MECAM was 10 times slower. Discrimination at the inner-membrane occurs in favor of the hydrolyzable triester backbones of enterobactin. Figure 4 provides a schematic of the siderophore mediated iron uptake systems in *E. coli*.²⁶

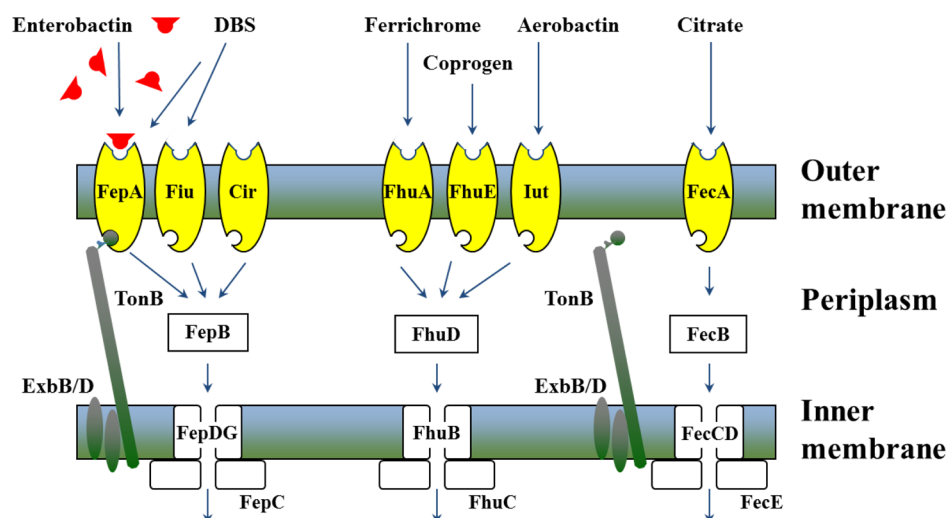


Figure 4. Siderophore uptake systems in *E. coli*. Siderophore uptake is both receptor and energy dependent. The outer membrane receptors are the most selective component of the systems. They have significantly different affinities or uptake rates for siderophores within the same class, for example, enterobactin and the enterobactin hydrolysis product, 2,3-dihydroxybenzoylserine (DBS).

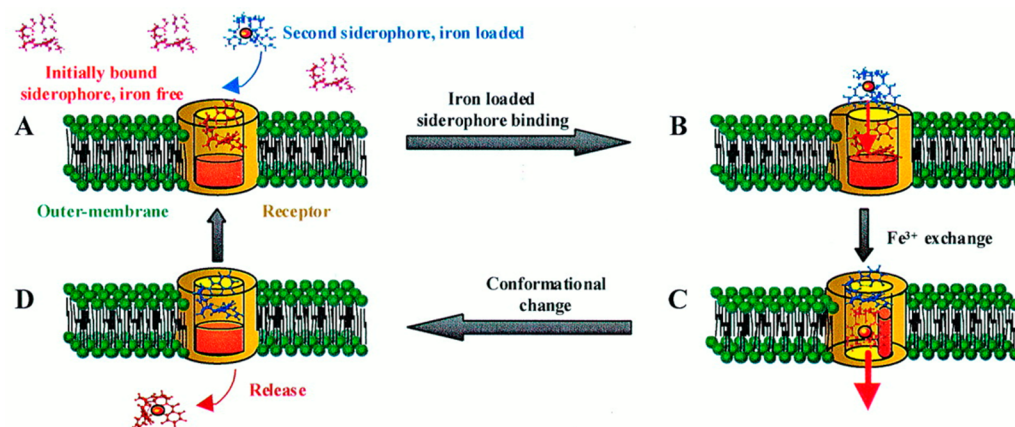


Figure 5. Proposed model of the siderophore shuttle iron exchange mechanism for iron transport in Gram-negative bacteria. (A) In vivo, apo-siderophore (red) may often be in excess of the ferric siderophore (blue), and thus the cognate receptor is predominantly loaded with the apo-siderophore. (B) A ferric siderophore approaches the receptor-bound apo-siderophore and transfers a ferric ion in a mechanism likely facilitated by the receptor. (C) Iron-binding by the siderophore inside the receptor barrel induces a conformational change that signals the iron-loaded status. Energized TonB then triggers translocation of the ferric siderophore to the periplasm. (D) Finally, the receptor returns to its initial conformation bound to an apo-siderophore. Reproduced with permission from ref 31. Copyright 2000 National Academy of Sciences, U.S.A.

Another technique used to study the effects of the coordination chemistry of siderophores on bacterial iron uptake involves substituting Fe(III) with another transition metal. While Cr(III) is similar to high-spin Fe(III) complexes in size and coordination geometry, Cr(III) complexes differ enormously in rates of ligand exchange. Our first use of Cr(III)–siderophores enabled transport studies to be carried out using radioactive labels with the assurance that loss of the metal had not occurred during the biological uptake process.

Kinetically inert chromium complexes of small molecule model hydroxamate analogs of ferrichrome and ferrioxamine B were prepared in addition to isolation of the natural siderophores from culture to characterize key physical characteristics of these compounds when complexed with iron.^{27–30} These studies included a collection of spectroscopic signatures, such as circular dichroism transitions and UV–visible bands, attributable to iron complexed by different moieties found in siderophores.

Later, inert Cr(III)–siderophore complexes were used to elucidate a siderophore transport mechanism called the siderophore shuttle. In this mechanism, observed in the Gram-negative bacteria *Aeromonas hydrophila* and *E. coli*, the outer membrane siderophore receptor is initially bound to an apo-siderophore (Figure 5).³¹ A second binding pocket of the receptor binds to a ferric siderophore. Then, in a required step, the metal is transferred from the ferric siderophore to the apo-siderophore, which is subsequently transported across the outer membrane. Inert Cr(III)–siderophore inhibited uptake, which supports the shuttle mechanism because Cr(III) exchange between an apo-siderophore and a Cr(III)–siderophore is prohibitively slow for this mechanism of metal uptake.

The salient features of the siderophore shuttle mechanism are apo-siderophore binding by the receptor and metal exchange. Apo-siderophore binding is expected to be detrimental to an iron uptake system unless the shuttle mechanism is present, but a survey of the biochemical data on siderophore receptors shows that many bind apo-siderophores

with high affinity, including several Gram-positive siderophore receptors. This characteristic suggests that these receptors may facilitate transport through a shuttle mechanism.

Much less is known about iron uptake by Gram-positive bacteria compared with Gram-negative bacteria. Because they have a single cell wall they present a different problem for the uptake mechanism compared with the Gram-negative bacteria with their two membranes. We focused on *Bacillus cereus* YxeB because it binds ferrioxamine, ferrichrome and the respective apo-siderophores with comparable affinity. In vitro and in vivo studies showed that iron binding and uptake are inhibited but not eliminated by Cr(III)–desferrioxamine. Metal exchange is facilitated by the receptor and increases the uptake rate of iron, but the YxeB system is not an obligate shuttle mechanism because a siderophore can be transported without a metal exchange step. A model for this observation is that transport by YxeB may proceed through two pathways, one that includes metal exchange and another that does not, and the metal exchange pathway is faster as illustrated in Figure 6.

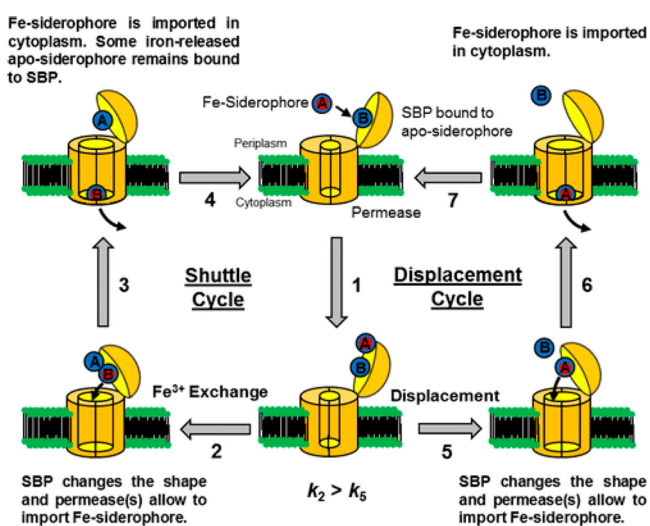


Figure 6. Models of the Gram-positive siderophore-shuttle mechanism and displacement mechanism of YxeB. YxeB is initially bound to an apo-siderophore. (1) An Fe-siderophore approaches YxeB and rests near the binding pocket occupied by the apo-siderophore. At this step, two pathways are possible. Steps 2–4 are the shuttle pathway. (2) Iron exchanges from the Fe-siderophore to the apo-siderophore in the binding pocket. The protein facilitates this step by increasing the local concentration of the entering ligand and the ferric complex. (3) The new Fe-siderophore (B) is transported and the created iron-released ligand (A) may remain bound by the YxeB protein. (4) The receptor is bound to an apo-siderophore. Steps 5–7 are the displacement pathway. (5) The Fe-siderophore displaces the apo-siderophore and occupies the binding pocket. (6) The original Fe-siderophore (A) is transported. (7) The SBP is bound to an apo-siderophore. In the Gram-positive siderophore-shuttle, both pathways operate but the shuttle pathway is preferred. Reproduced with permission from ref 32. Copyright 2014 American Chemical Society.

Observation of a siderophore shuttle mechanism in both Gram-positive and Gram-negative bacteria also suggests that this may be a general siderophore uptake mechanism with significant implications for the efficiency and diversity of siderophore-mediated iron uptake.

■ SIDEROPHORE THERMODYNAMICS

Early on it became apparent that the microbial competition for iron depended in an important way on the relative stability of the siderophore iron complexes, so studies were initiated to characterize the solution thermodynamic stability of these compounds.^{33,34}

Several investigations of fundamental siderophore properties focused on the archetypical siderophore enterobactin.²⁶ Enterobactin stands out among siderophores because it forms the most stable ferric complex of any siderophore known to date. The size, functional group arrangement, and electronic structure are optimal for binding ferric ion. Enterobactin binds iron with three catecholates arranged in near C_3 symmetry that branch from the aforementioned chiral triserine lactone backbone.

By measuring the properties of enterobactin analogs, we found that the structural features of enterobactin that most contribute to iron binding are the backbone, metal binding units, and hydrogen bonding of the catecholamides. The enterobactin backbone is a macrocycle that preorganizes the siderophore for metal binding, which in turn contributes entropically to the high stability of the ferric complex.^{35,36} The catechol metal binding units make a nearly optimal bite angle, the donor–metal–donor angle, for bidentate ligands with Fe(III).^{16,17} The catechols are adjacent to an amide functional group. The primary amide donates hydrogen bonds that stabilize the *ortho*-phenolate as observed in the few crystal structures of metal–enterobactin ($M = V(IV), Si(IV), Ge(IV), Ti(IV)$).^{36,37} The stabilization of the *ortho*-phenolate is crucial for metal binding at physiologically relevant pH levels.

Hydrogen ion concentrations impact metal binding because a metal and proton compete for the same binding sites. The catechol-amides in enterobactin stabilize the *ortho*-phenolates, lower the pK_a and reduce the proton competition for that site permitting the formation of stable complexes at physiological pH. Synthetic model chelators that do not have a hydrogen-bond-donating catechol-amide form stable complexes with iron only at high, nonphysiological pH values.

Below physiological pH, ferric enterobactin has three discrete protonation steps. The protonation constants ($\log K$) of the metal complex are 4.95, 3.52, and 2.5.³⁸ The protonation constants correspond to adding a proton at each of the three *meta*-phenolates. Protonation precludes iron coordination at the *meta*-phenolates and induces a change from the catecholate mode to a salicylate mode (Figure 7).^{39,40} This chelating

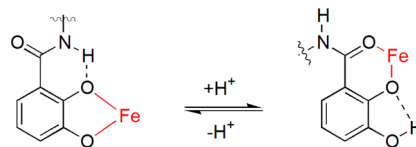


Figure 7. Catecholate (left) and salicylate (right) iron binding modes.

conformation allows all three binding units to remain coordinated to the metal even after three protonations, while many other siderophores release the metal after the first protonation. To adopt the salicylate mode, the amide rotates until the carbonyl is syn to the *ortho*-phenolate as observed by multiple spectroscopies. The metal–oxygen bond strengths are similar for both catecholate and salicylate complexes, but the ferric salicylate complexes are less stable than the catecholate complexes. The difference in stability is likely due to increased

strain in the carbon network of the siderophore because salicylate coordination forms a six-membered chelate ring in contrast to the five-membered ring of the catecholate mode.

Since the siderophore ligands differ in the denticity of their metal coordination and also in their relative acidities, metal stability constants are not directly comparable for all siderophores. These factors must be taken into account in calculating the relative affinity for stated solution conditions. This need led to the definition of the pM value,³³ a metric that continues to be in wide use today to compare the relative strength of metal chelators.

■ HOST–PATHOGEN COMPETITION FOR IRON

In the competition between bacteria and their hosts for iron, the rate as well as relative stability is important for iron exchange and bacterial uptake. Alvin Crumbliss and his co-workers have played a prominent role in elucidating siderophore exchange mechanisms.⁸ This in turn stimulated early investigations of iron removal kinetics by siderophores from human iron stores. Proteins such as transferrin, ovotransferrin, and lactoferrin are responsible for delivering iron throughout the human body and are potentially sources of iron for invading bacterial pathogens. Each of these proteins has the potential to bind two iron atoms at any given time. However, the release of iron from each of these sites differ significantly. In human transferrin, the N-terminal and C-terminal domains each bind iron with a synergistic anion (carbonate) and similar residues. However, the channel leading to the iron binding site in each lobe is different. The C-terminal channel is concealed, and accessibility of this channel is dependent on the conformation of the N-terminal binding domain. The N-terminal channel is exposed and thus serves as a potential opening for iron removal by small molecule chelators. However, some chelators preferentially remove iron from the C-terminal domain of transferrin, despite the N-terminal being more exposed to the surrounding environment.^{41,42} Different chelators remove iron from transferrin by different mechanisms, because iron removal is dependent on the pH and the ability of a given chelator to displace the synergistic bicarbonate ion and to induce a conformational change in the protein lobes.

The human body employs several regulatory systems to protect against invading iron-requiring pathogens. Heparin⁴³ triggers a decrease in iron export, increase in iron storage in cells, and increased concentration of apo iron-binding proteins, which in turn disrupt microbial iron metabolism. A major change in the understanding of the competition of pathogenic bacteria for human iron stores has occurred in the last 13 years with the characterization by Professor Roland Strong of the protein now generally called siderocalin. This human protein is a product of the human innate immune system.⁴⁴ It binds with high affinity to many catecholate siderophores in both the apo and iron bound forms (Figure 8). Our studies^{45–47} revolved around siderocalin and its interaction with iron–siderophores. Siderocalin limits bacterial iron uptake by binding siderophores. This defense strategy is effective in protecting against many infections. The siderophore binding pocket rests within the calyx of an eight-stranded antiparallel β -barrel. Three basic residues project into the binding pocket giving it a positive charge. Siderocalin binds ferric enterobactin with high affinity through Coulombic and cation– π interactions.⁴⁸ The three positive residues define three subpockets into each of which fits a catecholate unit of enterobactin. By complementing the metal center of enterobactin, the pseudo-3-fold symmetry and



Figure 8. Siderocalin, the first human protein found to specifically bind siderophores. Reproduced with permission from the cover of *Molecular Cell*, vol 10, iss 5. Copyright 2002 Elsevier.

cationic interaction provided by the binding pocket also recognizes many other triscatecholate siderophores with high affinity. In addition to enterobactin, siderocalin binds bacillibactin, parabactin, carboxymycobactin, fluvibactin, and vibriobactin. The binding pocket degeneracy enables siderocalin to defend against siderophore mediated iron acquisition from a variety of siderophores produced by pathogens. However, several pathogens have responded to the siderocalin defense by modifying the siderophores to block binding by the host siderocalin. These “stealth” siderophores include petrobactin and the salmochelins.^{49,50} Figure 9 shows how the

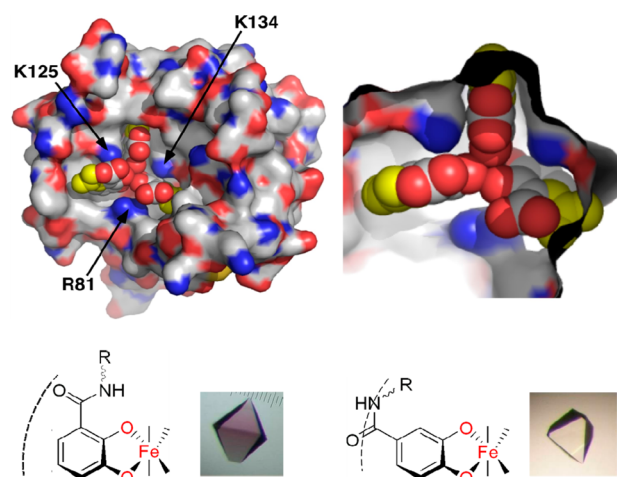


Figure 9. Siderocalin binding of 2,3-catechol amides versus 3,4-catechol amides. Shown at upper left is the protein calyx and its interaction with the iron catechol complex, with the detail shown at upper right. Below are shown the metal complexes and resultant binding by the protein. The intense red is due to the bound ferric complex, which is absent at lower right. Reproduced with permission from ref 49. Copyright 2006 National Academy of Sciences, U.S.A.

altered structure of the petrobactin catechol groups precludes binding by siderocalin. The discovery that there are “stealth” siderophores explained a long-standing puzzle. *E. coli* produces two primary siderophores: enterobactin and aerobactin. The first is a catecholate with high iron affinity and the primary siderophore for our intestinal symbionts. The second is a weaker hydroxamate-citrate chelator produced by virulent strains. In serum circulation, enterobactin is inactivated by siderocalin, whereas aerobactin is not. The interplay between “stealth” siderophores and the host immune system have been reviewed elsewhere.^{6,7,39,45,47,51}

Although it had long been speculated that there might be human siderophores (i.e., low molecular weight chelators that play an iron transport role in humans), none had been found. However, siderocalin can play an iron transport role, especially in early mammalian embryo development, and siderocalin is expressed 10–500 times more in serum and urine after damage stimuli (bacterial and nonbacterial). Then it was found that catechol is an endogenous siderophore.⁵² Mouse urine contains several catechols in relatively high concentrations. Catechol itself was found to be present at about 0.2 μM . The strong binding of the catechol–iron complexes by siderocalin drives formation of the 2:1 or 3:1 complexes. Siderocalin and catechol introduced separately in vivo sequester iron and form the ternary siderocalin–iron–catechol complex. Subsequently iron is predominantly delivered to kidney cells, completing the iron transport role. It was later claimed that gentisic acid or 2,5-dihydroxybenzoic acid is a mammalian siderophore candidate and induces cellular iron efflux and apoptosis.⁵³ This seemed unlikely, since the catechol groups of this molecule are trans to each other and cannot form a metal chelate ring. Indeed, a detailed study later reported that 2,5-dihydroxybenzoic acid is not a mammalian siderophore.⁵⁴

In Gram-positive bacteria, iron–siderophores are actively internalized through ABC-type transporters, which are composed of three components: a substrate or siderophore binding protein (SBP), channel, and an ATPase. The SBP is the first site of recognition and selectivity for a siderophore or iron–siderophore. Typically SBPs discriminate for particular siderophores based on iron binding moieties, siderophore, or iron–siderophore size, shape, or chirality. Some SBPs are more promiscuous than others, allowing siderophores synthesized from other bacteria, or so-called xenosiderophores, to be imported. In this way bacteria conserve energy by avoiding the cost of siderophore biosynthesis.

The *Bacillus cereus* group of bacteria includes some extremely pathogenic species such as *Bacillus cereus*, itself a potentially enterotoxigenic pathogen in humans, and *Bacillus anthracis*, the lethal anthrax pathogen. Members of this group can synthesize and secrete the siderophores bacillibactin and anthrax virulence-associated petrobactin. The SBPs of *B. cereus* directly implicated in petrobactin uptake were identified as FpuA and FatB through a combination of ⁵⁵Fe–siderophore uptake studies and fluorescent binding assays.⁵⁵ Furthermore, we showed that QTOF ESI-MS is a useful tool to characterize noncovalent interactions between siderophore or siderophore–metal complexes and recombinantly expressed proteins. FatB was shown to bind a select set of petrobactin-related compounds such as the precursor and petrobactin photoproduct, 3,4-dihydroxybenzoic acid, in addition to petrobactin. FpuA was shown to be specific for apo and ferric PB.

The petrobactin-binding protein in *B. subtilis*, another member of the *B. cereus* group, was identified as YclQ and

characterized by crystallography at 1.75 Å resolution.⁵⁶ According to modeled ferric siderophore structures in the YclQ binding domain, the YclQ residues Glu-107, Lys-84, and His-214 are suspected to be responsible for the specific binding of YclQ to substrates. The size of the binding pocket is also large enough to accommodate siderophores such as Fe(PB). Zawadzka et al.⁵⁶ quantified YclQ affinity for various siderophores using a fluorescence binding assay in which the inherent protein fluorescence is quenched upon the addition of a strong binding siderophore. YclQ specifically binds 3,4-catechol siderophores such as petrobactin and 3,4-dihydroxybenzoic acid, whereas its affinity for 2,3-catechol siderophores is orders of magnitude weaker and suggests nonspecific binding. The Fe(PB) is photolabile because the α -hydroxycarboxylate, when bound to iron, is photoactive and can decarboxylate to the petrobactin photoproduct (PB ν).⁵⁶ Zawadzka et al. showed that Fe(PB ν) has an even lower affinity for YclQ than Fe-PB, which may hint at an iron release mechanism dependent on redox activity.

Citrate is a nearly ubiquitous biomolecule and an iron chelator and widely used siderophore. We identified new ferric citrate uptake machinery in pathogenic *B. cereus* that is different from the YfmCDEF SBP in *B. subtilis*.⁵⁷ A ⁵⁵Fe-citrate radiotracing experiment showed that the *B. cereus* strain ATCC 14579 imports ferric citrate. The siderophore-binding protein of this system was isolated and named ferric citrate-binding protein C (FctC) after protein fluorescence quenching assays and nano-ESI-MS analyses demonstrated the specificity of FctC for the ferric citrate species. Nano-ESI-MS results consistently showed the formation of the ternary FctC/FeIII/citrate complex across a variety of pH values and ratios of Fe/cit. The iron citrate species that are bound by FctC are either Fe₃cit₃ or Fe₂cit₂. The affinity of FctC for ferric citrate was determined by fluorescence quenching assays and subsequent nonlinear regression analyses, which gave a protein–ligand dissociation constant (K_d). Specifically, the nano-ESI-MS results suggest that ferric citrate complexes of iron/citrate ratios 3:3 and 2:2 are compatible ligands that are bound with high nanomolar affinity to FctC. The calculated K_d of FctC for ferric citrate trimer is one of the lowest reported for a siderophore-binding protein and a given iron-containing ligand.

The remarkable ability of FctC to fish out the iron citrate trimer, a species of low abundance, was consistent across changes in pH and variation of the molar ratio of Fe/cit. The *B. cereus* bacteria may encounter various environments in which the pH and concentrations of iron and citrate are not optimal. Perhaps the binding of the iron citrate trimer is advantageous to the bacterium. The FctC selectivity could be part of *B. cereus*' recognition mechanism for iron citrate and could potentially be the discriminatory site in which other metal–citrate species in the surrounding environment are not bound and consequently not imported. The proposed ferric citrate trimer is shown in Figure 10.

CONCLUSION

In a 42 year effort of studying siderophore mediated iron transport, the focus has widened from simply studying the coordination chemistry of these small molecules to their role in vivo as both iron delivery agents for bacteria and agents in the host/pathogen arms race for iron. Siderocalins are a key part of this competition. There are now many siderocalins from several species that have been characterized. Some of these are very different in primary structure and hint at convergent evolution.

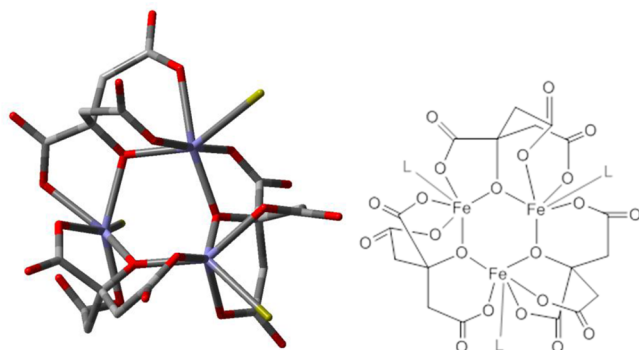


Figure 10. *Bacillus cereus* uses ferric citrate as one vehicle for iron delivery. FctC is a ferric-citrate siderophore binding protein of *B. cereus*; it binds Fe_3Cit_3 ($K_d = 0.27$ nM), although this is a trace species in solution. The structural model proposed has all carboxylate oxygens coordinated to the Fe(III) center (red is O, blue is Fe). The remaining ligands are from the protein receptor. Adapted with permission from ref 57. Copyright 2012 National Academy of Sciences, U.S.A.

They also seem to target the siderophores of that particular species' primary pathogens. It will be interesting to see whether this speculation is confirmed.

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Notes

The authors declare no competing financial interest.

Biographies

Kenneth N. Raymond, Chancellor's Professor at the University of California, Berkeley, was born in Astoria, Oregon, in 1942. He attended Reed College, where he received a B.A. in 1964. His Ph.D. research at Northwestern University, under the direction of Professors Fred Basolo and James A. Ibers, concerned the synthesis and structure of five-coordinate metal complexes. Upon completing his Ph.D., he began his faculty appointment at the University of California at Berkeley in 1967, becoming Associate Professor in 1974 and Professor in 1978. His work has been recognized with several awards, including the Ernest O. Lawrence Award of the Department of Energy (1984), a Humboldt Research Award for Senior U.S. Scientists (1991), and the American Chemical Society Alfred Bader Award in Bioinorganic or Bioorganic Chemistry (1994). He has been an Alfred P. Sloan research fellow (1971–1973), a Miller research professor at the University of California (1977–1978, 1996), and Guggenheim fellow (1980–1981). He was elected to the National Academy of Sciences in 1997 and the American Academy of Arts and Sciences in 2001. In 2008, Professor Raymond was honored with the ACS Award in Inorganic Chemistry. In addition to his academic appointment, he is a cofounder of Lumiphore Inc., which utilizes new luminescent agents developed in his laboratory, and Faculty Senior Scientist at Lawrence Berkeley National Laboratory.

Benjamin E. Allred was born in Provo, Utah, in 1984 and was raised in Idaho. He received his B.S. from Brigham Young University and his Ph.D. from UC Berkeley in 2013 under the direction of Kenneth Raymond. He carried out postdoctoral studies at Lawrence Berkeley National Laboratory with Rebecca Abergel, and he is currently a postdoctoral researcher with Daniel Herschlag at Stanford University. His research has focused on understanding the properties and consequences of metal ion–biomolecule interactions.

Allyson K. Sia was born in Waukegan, IL, in 1985. She earned her B.S. from the University of California, Irvine, in 2008 and conducted undergraduate research under the guidance of Prof. Alan F. Heyduk investigating redox active ligands for early transition metal catalysts. She obtained her Ph.D. from the University of California, Berkeley, in 2013 under the leadership of Professor Kenneth N. Raymond studying siderophore-mediated iron transport in the context of pathogenic bacteria and humans. Allyson is currently a scientist at Amgen, Inc., evaluating the safety of protein therapeutics for patients.

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