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## Beyond iron: non-classical biological functions of bacterial siderophores

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Bacteria secrete small molecules known as siderophores to acquire iron from their surroundings. For over 60 years, investigations into the bioinorganic chemistry of these molecules, including fundamental coordination chemistry studies, have provided insight into the crucial role that siderophores play in bacterial iron homeostasis. The importance of understanding the fundamental chemistry underlying bacterial life has been highlighted evermore in recent years because of the emergence of antibiotic-resistant bacteria and the need to prevent the global rise of these superbugs. Increasing reports of siderophores functioning in capacities other than iron transport have appeared recently, but reports of "non-classical" siderophore functions have long paralleled those of iron transport. One particular non-classical function of these iron chelators, namely antibiotic activity, was documented before the role of siderophores in iron transport was established. In this Perspective, we present an exposition of past and current work into non-classical functions of siderophores and highlight the directions in which we anticipate that this research is headed. Examples include the ability of siderophores to function as zincophores, chalkophores, and metallophores for a variety of other metals, sequester heavy metal toxins, transport boron, act as signalling molecules, regulate oxidative stress, and provide antibacterial activity.

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## Introduction

Iron is an essential nutrient for nearly all known life forms. The caveat is included in the preceding statement because

certain lactobacilli and the causative agent of Lyme disease, *Borrelia burgdorferi*, do not require iron for growth.<sup>1,2</sup> Iron is commonly used in biological systems most likely because of its Earth abundance and the breadth of the chemistry that it can undergo.<sup>3</sup> This element exists in two readily inter-convertible oxidation states: ferrous and ferric, or Fe(II) and Fe(III), respectively. The ability to convert between these two states allows iron to play a pivotal role in numerous electron transfer

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Timothy Johnstone completed his B.Sc. (Hons) studies at McGill University in 2009, where he worked under the guidance of Professors Bruce Arndtsen, Scott Bohle, Jay Nadeau, and Derek Gray. He subsequently carried out his doctoral research under the supervision of Professor Stephen Lippard at the Massachusetts Institute of Technology. Following completion of his thesis on the chemistry and nanodelivery of platinum anti-

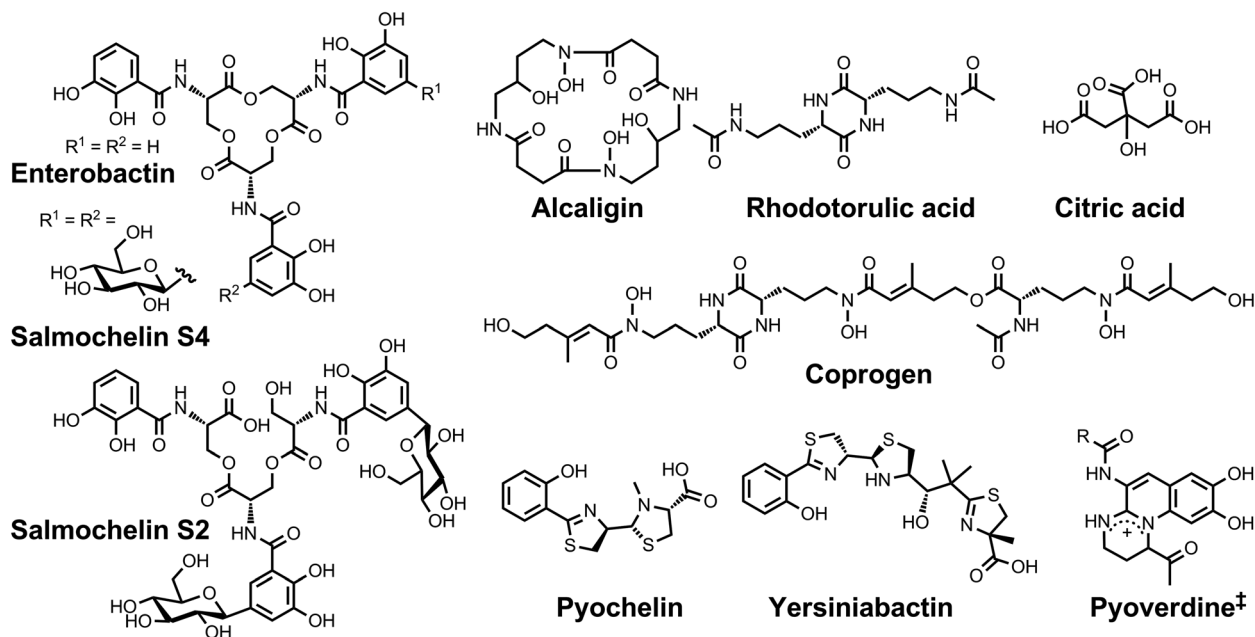
cancer agents in 2014, he joined the laboratory of Professor Elizabeth Nolan at MIT. There, he is working on siderophore-antibiotic conjugates for antibacterial applications.



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**Chart 1** Chemical structures of the siderophores discussed in the introduction. The stereochemistry of yersiniabactin was taken from ref. 31. The structure of salmochelin S2 is that reported in ref. 32. An alternative structure of a linearized salmochelin S4 in which a glucosyl unit is attached at the carboxylic acid end of the linearized trimer instead of the alcohol end has also been reported.<sup>33</sup> The enantiomer of the form of pyochelin shown, *enantio*-pyochelin, is a known siderophore. †Many pyoverdines exist and are distinguished by the nature of the peptide chain attached at the R position.

processes.<sup>4</sup> The precisely tuned steric and electronic environments within enzyme active sites permit access to more highly oxidized, Fe(IV) and Fe(V), or reduced, Fe(I), states that are essential intermediates in catalytic biochemical transformations that range from methane oxidation to proton reduction.<sup>5,6</sup> Moreover, iron centres can access different spin states, which allows chemical reactions to occur at this metal centre that are not as readily feasible in purely organic systems.<sup>7,8</sup> Despite the ubiquity of iron in biology, the very nature of its aqueous chemistry dictates that, under aerobic conditions and at neutral pH, the concentration of dissolved iron is lower than the concentration needed to sustain bacterial life.<sup>9</sup> In this regard, and in the discussions below, dissolved or soluble iron refers to solvated aqua/hydroxo complexes of the metal.

The solubility of Fe(III) in water at neutral pH is typically quoted to be  $10^{-18}$  M,<sup>10,11</sup> a value derived from the solubility product of Fe(OH)<sub>3</sub>,  $K_{sp} \approx 10^{-39}$ ,<sup>12</sup> and the concentration of hydroxide at neutral pH,  $[OH^-] = 10^{-7}$  M. An alternative soluble Fe(III) concentration of 1.4 nM has been proposed on theoretical grounds following re-evaluation of the importance of Fe(OH)<sub>2</sub><sup>+</sup> (aq.) in the speciation of this element.<sup>13,14</sup> Regardless of this correction, the concentration of Fe(III) in solution falls far below the level to which iron is concentrated within bacteria. For instance, spectrochemical analysis revealed the dry weight of *Escherichia coli* to be 0.021% Fe.<sup>15</sup> A cell volume of  $10^{-15}$  L<sup>16,17</sup> and a dry mass of  $10^{-13}$  g<sup>17</sup> affords a whole cell concentration of Fe that is approximately  $10^{-3}$  M. This concentration deviates from the value previously quoted ( $10^{-6}$  M);<sup>11</sup>

however, a comparison of either value to the solubility of Fe(III) highlights the extent to which bacteria concentrate Fe obtained from the environment. For commensal and pathogenic bacteria that colonize humans, iron concentrations are even more limited. The low levels of Fe(III) that exist freely in solution are toxic to mammals and are accordingly suppressed by a number of mechanisms. Most notably, Fe(III) levels in human serum are maintained at approximately  $10^{-24}$  M by transferrin,<sup>18</sup> an abundant iron transport protein. We note that this concentration of Fe(III) in serum is obtained by using the transferrin  $\log K_1 = 22.7$ ,  $\log K_2 = 22.1$ , and the estimation that at any given time about 30% of the binding sites of transferrin in blood are empty.<sup>18</sup>

In order to acquire iron under these conditions, bacteria employ a number of transport mechanisms.<sup>10</sup> One of the most striking is the secretion of *siderophores*, small molecules that exhibit high binding affinity for iron.<sup>19</sup> A similar strategy is employed by fungi<sup>20</sup> and plants<sup>21</sup> but the focus of this Perspective will be on bacterial siderophores. These chelators exhibit proton-independent stability constants ( $\beta$ ) for iron complexation ranging from approximately  $10^{10}$  to  $10^{49}$ .<sup>22,23</sup> The latter is the  $\beta_{110}$  value for the complex of Fe(III) and enterobactin<sup>6-</sup>, where the  $\beta_{MLH}$  notation indicates the formation constant for a given stoichiometry of metal (M), fully deprotonated ligand (L), and protons (H). Although the  $\beta$  values of different siderophores may not be directly comparable because of differences in ligand  $pK_a$  values and metal : ligand binding ratios, enterobactin is widely credited as the siderophore with the highest known affinity for iron.<sup>24</sup> This statement is borne out by



**Table 1** Putative non-classical biological functions of the bacterial siderophores discussed in this Perspective

Siderophore	Non-classical function(s)	Chart(s) containing structure
Aerobactin	—	6
Alcaligin	Cell signalling	1, 6
Aminochelin	Mo/V transport	3
Azotobactin	Mo/V transport	3
Azotochelin	Mo/V transport	3
Citrate	—	1
Coelibactin	Zn binding	2
Coelichelin	Zn binding	2
Coprogen	—	1
Danoxamine	Antibiotic component	7
Delftibactins (A, B)	Other non-iron metallophore	5
(Des)ferrioxamine B	Other non-iron metallophore	5
(Des)ferrioxamine E	Other non-iron metallophore	5
2,3-Dihydroxybenzoylserine	Cell signalling	6
Enterobactin	Cell signalling	1, 4, 6, 7
	Oxidative stress response	
Ferrichrome	Antibiotic component	7
Micacocidin	Zn binding	2
Protochelin	Mo/V transport	3
	Other non-iron metallophore	
Pyochelin	Cu binding	1, 4
	Other non-iron metallophore	
	Antibiotic activity	
Pyoverdines	Zn binding	1, 2, 4, 6, 7
	Cu binding	
	Other non-iron metallophore	
	Cell signalling	
Pyridine-2,6-dithiocarboxylate (pdtc)	Zn binding	2, 4
	Cu binding	
	Other non-iron metallophore	
Rhizoferrin	Zn binding	2
Rhodotorulic acid	—	1
Salmochelins (S2, S4)	Oxidative stress response	1, 7
Schizokinen	Cu binding	4
	Other non-iron metallophore	
Staphyloferrin A	Oxidative stress response	7
Staphyloferrin B	Oxidative stress response	7
Vibrioferriin	Boron transport	5, 6
	Cell signalling	
Yersiniabactin	Zn binding	1, 2, 4, 6
	Cu binding	
	Cell signalling	
	Oxidative stress response	

metrics, such as  $p\text{Fe(III)}$ ,<sup>†</sup> which more accurately compare the stabilities of  $\text{Fe(III)}$  complexes under physiologically relevant conditions.<sup>24</sup> The enormous range in stability constants reflects the diversity of chemical composition across the >260 siderophores of known chemical structure.<sup>25,26</sup> The chemical structures of the siderophores discussed in this paper are collected in Charts 1–9. The chemical structures and stereochemical assignments are in general agreement with those given in a recent comprehensive review of the siderophore literature,<sup>26</sup> and when differences occur they are noted in the Chart captions. Table 1 lists all of the siderophores discussed in this Perspective in alphabetical order, provides the locations of the corresponding chemical structures, and summarizes the non-classical functions of each.

<sup>†</sup> The  $p\text{Fe(III)}$  is the negative of the decadic logarithm of the concentration of free  $\text{Fe(III)}$  in solution under the following fixed conditions:  $\text{pH} = 7.4$ , total ligand concentration = 10  $\mu\text{M}$ , and total  $\text{Fe(III)}$  concentration = 1  $\mu\text{M}$ .

The typical metal-binding motifs – catecholates, hydroxamates, and  $\alpha$ -hydroxycarboxylic acids – select for  $\text{Fe(III)}$  in accordance with the hard-soft acid–base theory. These hard oxygen-atom donors are well-matched to the hard ferric ion. Many siderophores present six coordinating atoms to the metal centre in a pseudooctahedral geometry and a number of these ligands exhibit a metal-binding pocket that is preorganized, typically *via* macrocyclization. For example, the linear dihydroxybenzoylserine trimer, a product of the hydrolysis of enterobactin, exhibits a proton-independent formation constant of  $10^{43}$ , as compared to the cyclized parent compound, which exhibited a corresponding  $\beta$  value of  $10^{49}$ .<sup>27</sup> Additionally, the macrocyclic dihydroxamate siderophore alcaligin binds  $\text{Fe(III)}$  32 times more strongly than the linear dihydroxamate rhodotorulic acid, as determined by comparing the proton-independent stability constants of the 1 : 1  $\text{Fe}$ –siderophore complexes.<sup>28</sup> In addition to macrocyclization, another general strategy employed in siderophore biosynthesis is heterocyclization, exemplified by the enzyme-catalyzed cyclodehy-



dration of X-cysteine and X-serine dipeptide motifs to form thiazoline and oxazoline rings, respectively.<sup>29</sup> These heterocycles can undergo further modifications, including dehydration and aromatization to afford thiazoles and oxazoles, or reduction to afford thiazolidines and oxazolidines. Such chemistry, used in the biosynthesis of siderophores such as yersiniabactin and pyochelin, rigidifies the peptide backbone and increases the basicity of the formerly peptidic nitrogen atom, allowing it to better coordinate iron.<sup>30</sup>

Many bacteria produce, or are capable of utilizing, multiple siderophores. For instance, the laboratory strain *E. coli* K-12 expresses machinery for the uptake of ferric complexes of enterobactin, linearized enterobactin, citrate, ferrichrome, rhodotorulate, and coprogen.<sup>24</sup> *E. coli* K-12 cannot produce the latter three siderophores, however.<sup>34</sup> Siderophores used by a non-producer organism are called *xenosiderophores*. The FoxA-mediated uptake of ferrioxamine by *Yersinia enterocolitica* and the uptake of enterobactin via *Pseudomonas aeruginosa* PfeA and PirA are further examples of *xenosiderophore* utilization.<sup>35–37</sup> The ability of organisms to use redundant siderophore-mediated iron uptake pathways has prompted the question as to why such overlap exists.

One answer to this question is that different siderophores can better extract iron from different sources or under different environmental conditions.<sup>38–40</sup> Additionally, certain siderophores permit bacterial pathogens to better evade the host immune system. For example, *Salmonella enterica* produce enterobactin to satisfy their nutritional requirements, but humans have evolved a defence mechanism whereby the secreted host-defence protein lipocalin-2 binds and sequesters ferric enterobactin. In response, *Salmonella* produce salmochelins,<sup>41</sup> enterobactin derivatives that are glucosylated at the C5 position of one or more catechol rings to prevent capture by lipocalin-2.<sup>42</sup> Certain pathogenic *E. coli* also produce salmochelins to overcome the host response to infection.<sup>41</sup> *P. aeruginosa* strains typically produce at least two siderophores, pyoverdine and pyochelin.<sup>43</sup> Pyoverdines coordinate Fe(III) with high affinity (for pyoverdine PaA,  $K_a = 10^{30.8}$  and  $pK_a = 27$ ),<sup>44</sup> whereas pyochelin exhibits much lower affinity ( $K_a = 10^{17.3}$  and  $pK_a = 16$ ).<sup>45</sup> Mathematical simulations support the hypothesis that *P. aeruginosa* strains with the capacity to switch between producing and utilizing these two siderophores in response to changing iron conditions have an enhanced degree of fitness over those that cannot.<sup>46</sup>

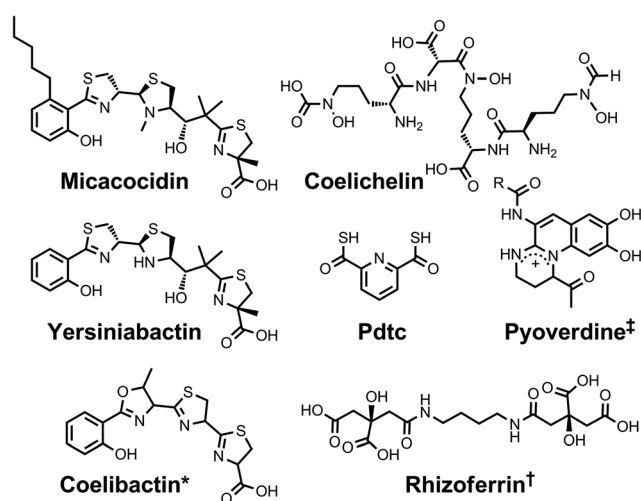
Although access to multiple siderophores can confer advantages related to iron acquisition, another logical proposal is that organisms may produce seemingly redundant siderophores because some of these molecules are actually serving functions other than iron delivery. Indeed, investigations into alternative, non-classical functions of siderophores have revealed that this scenario occurs. Studies approaching this problem from both chemical and biological perspectives have revealed novel bioinorganic chemistry. In this Perspective, we intend to provide the reader with an overview of non-classical siderophore functions and the connections that exist between them (Table 2). We also intend for this exposition to spark new initiatives that will address

as-yet unconceived siderophore functions. We will limit our discussion to biological siderophore functions, and we refer the reader to a number of reviews on the technological potential of siderophores, which, although arguably distinct from the classical siderophore function, fall outside the scope of this Perspective.<sup>47–50</sup>

In the following sections, we will begin with a non-classical function that is perhaps the simplest extension from iron transport: the transport of other essential metals such as zinc, manganese, molybdenum, and vanadium. The interaction of siderophores with copper and heavy metals will also be discussed. A digression from metal-siderophore interactions will summarize the role that marine siderophores may play in boron uptake. The focus will then shift from siderophores binding ions and influencing mass transport to the putative role of metal-siderophore complexes and apo siderophores as signalling molecules. The ability of siderophores to protect bacteria from oxidative stress will also be addressed. We will conclude with a return to ferric siderophore complexes and present sideromycins, antibiotic-siderophore conjugates that exploit bacterial iron uptake machinery to enhance efficacy. Investigations into this non-classical siderophore function may prove to be particularly valuable in the development of therapies targeting the antibiotic-resistant pathogenic bacteria that are infesting hospitals worldwide.

### Siderophores as zincophores

Following iron, zinc is the most abundant transition metal in *E. coli*.<sup>15</sup> It has been estimated that approximately 5% of the *E. coli* proteome comprises zinc-binding proteins.<sup>51</sup> Given the important structural and catalytic roles that Zn(II) plays in bac-



**Chart 2** Chemical structures of the siderophores discussed in *Siderophores as Zincophores*. The stereochemistry of yersiniabactin was taken from ref. 31. \*The structure of coelibactin is tentative and based on bioinformatics analyses; stereochemistry has yet to be established.<sup>56</sup> †Rhizoferrin can occur as shown (S,S) or in the enantiomeric (R,R) form. ‡Many pyoverdines exist and are distinguished by the nature of the peptide chain attached at the R position. Pdtc is pyridine-2,6-dithiocarboxylic acid.





terial proteins, it is not surprising that these organisms have machinery, such as the ZnuABC transporter, dedicated to zinc uptake.<sup>52</sup> The importance of Zn(II) acquisition for bacterial pathogens is underscored by the fact that humans secrete zinc-sequestering proteins (e.g. calprotectin and psoriasin) to inhibit the progression of microbial infections.<sup>53,54</sup> It is possible that zinc-withholding by the host applies an evolutionary pressure on pathogenic bacteria to develop high-affinity zinc uptake mechanisms akin to those in which siderophores play a role.<sup>55</sup> Little is known, however, about the extent to which bacteria secrete high-affinity zinc chelators, termed *zincophores* or *tsinkophores*.<sup>54</sup>

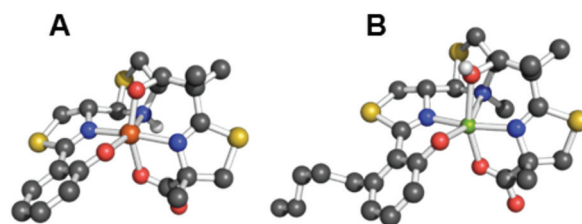
After the genome of the filamentous soil-dwelling bacterium *Streptomyces coelicolor* was sequenced,<sup>57</sup> two non-ribosomal peptide synthases (NRPSs) were identified and deduced to be responsible for the biosynthesis of two small molecules, namely coelichelin and coelibactin. The structures of these molecules were predicted on the basis of bioinformatics analyses, which included identification of the specificity-determining residues of the NRPS adenylation domains that select and activate monomeric precursors.<sup>58</sup> Subsequently, the proposed structure of coelichelin was amended following NMR studies of its gallium complex.<sup>59</sup> Comparison with known siderophore structures suggested that coelichelin and coelibactin also function in iron transport. During later studies on the effect of zinc on the production of antibiotics by *S. coelicolor*, it was observed that increased zinc levels decreased the expression of coelibactin, as determined by qRT-PCR of the coelibactin NRPS mRNA transcripts, and a putative zincophore activity was ascribed to this molecule.<sup>60</sup> Next, a study of the effect of coelibactin on *S. coelicolor* sporulation confirmed that Zur, the zinc uptake repressor, regulates coelibactin biosynthesis.<sup>61</sup> Although the biosynthesis of coelibactin has been investigated,<sup>56,62</sup> this metallophore has not yet been isolated from *S. coelicolor* and structurally elucidated; thus, its coordination chemistry remains to be explored.

A variety of pseudomonads produce the small molecule metal chelator pyridine-2,6-dithiocarboxylic acid, or pdtc, which was first identified as a result of its ability to bind iron and was therefore described as a siderophore.<sup>63</sup> Production of this molecule has since been observed in both environmental samples and in laboratory cultures of *Pseudomonas stutzeri* KC grown either aerobically or anaerobically.<sup>64–66</sup> The soft character of the sulphur donor atoms of pdtc favours complex formation with soft metals, providing higher affinity for Fe(II) than Fe(III). Pdtc also complexes zinc,<sup>67</sup> and the ability of pdtc to effect biological transport of zinc was demonstrated in *Pseudomonas putida*.<sup>68</sup> Subsequent studies revealed that the zinc-pdtc complex is recognized and transported by the outer membrane receptor and inner membrane permease of the pdtc utilization machinery; however, transport of zinc-pdtc is much less efficient than transport of the iron-pdtc complex.<sup>69</sup>

Very recently, it has been demonstrated that yersiniabactin, a heterocyclic siderophore produced by *Yersinia pestis* and some pathogenic *E. coli*, can act as a zincophore.<sup>70</sup> The ability

of *Y. pestis* *znu* mutants lacking functional ZnuABC transport machinery to grow in response to supplementation of Chelex-treated medium with zinc suggested that an unidentified high-affinity zinc transporter was operational.<sup>71</sup> In support of this proposition, none of the other divalent metal ion transport systems of this organism mediated zinc uptake.<sup>71,72</sup> Mutations in the *irp2* gene encoding the yersiniabactin synthetase HMWP2 produced severe growth defects when combined with a  $\Delta znuBC$  mutation. Bacterial growth was restored by either complementation with the *irp2* gene *in trans* or Zn(II) supplementation.<sup>70</sup> Moreover, addition of purified exogenous apo-yersiniabactin stimulated growth in mutant *Y. pestis* strains lacking a functional ZnuABC system. Remarkably, uptake of the yersiniabactin-complexed zinc requires neither the outer membrane receptor nor the inner membrane ABC transporter that are required for yersiniabactin-mediated iron transport, indicating that a separate uptake pathway is operative.<sup>72</sup> The interaction of yersiniabactin with Zn(II) may have important implications for human disease based on experiments in a mouse model of septicemic plague. The ZnuABC transport system and HMWP2, likely *via* its role in yersiniabactin synthesis, both contribute to the development of lethal *Y. pestis* infections.<sup>70</sup>

Explicit studies of the zinc coordination chemistry of yersiniabactin appear to be lacking in the literature. In this context, we note that the antibiotic micacocidin, produced by *Pseudomonas* sp. No. 57–250, complexes zinc as well as iron and copper.<sup>73,74</sup> The structure of micacocidin is identical to that of yersiniabactin with the exception of methylation of the nitrogen atom in the saturated thiazolidine ring and substitution of the phenolic ring at the 3-position with an *n*-pentyl group. Micacocidin forms a complex with Fe(III), known as micacocidin C, but was not investigated as a siderophore until recently.<sup>75</sup> In addition to the Fe(III) complex, a Cu(II) complex, micacocidin B, and a zinc complex, micacocidin A, were isolated from bacterial cultures.<sup>73</sup> Micacocidin A was the dominant product obtained from bacterial culture and was characterized crystallographically.<sup>74,76</sup> The crystal structure reveals a coordination geometry resembling that of ferric-yersiniabactin (Fig. 1).<sup>31</sup> One key difference is that the deprotonated secondary alcohol in the structure of the Fe(III) complex remains protonated in the Zn(II) structure, resulting in charge neutrality for both compounds. The Zn(II)-bound structure of micacocidin corroborates a



**Fig. 1** Ball-and-stick representations of the metal complexes from the crystal structures of (A) yersiniabactin–Fe(III) (CCDC ID: 619878)<sup>31</sup> and (B) micacocidin–Zn(II) (micacocidin A, CCDC ID: 130920).<sup>76</sup> Non-polar hydrogen atoms have been omitted for clarity. Colour code: C gray, O red, N blue, H white, Fe orange, and Zn green.



zinc-related non-classical function for yersiniabactin, and highlights the need for detailed investigations of the zinc coordination chemistry of the latter siderophore.

### The interaction of siderophores with manganese

Manganese is another essential nutrient and, in prokaryotes, Mn-containing metalloproteins carry out functions ranging from photosynthesis to oxidative stress defence.<sup>77</sup> The metal-withholding host-defence paradigm described above in relation to iron and zinc extends to manganese as well.<sup>53</sup> Bacterial protein-based uptake systems for manganese have been described in Gram-negative and -positive strains, and further studies continue to reveal how these proteins contribute to manganese homeostasis.<sup>78,79</sup> The bacterial manganese transport protein MntC and its homologues are extra-cytoplasmic solute-binding proteins that bind Mn(II) and interact with other members of the ABC transporter to bring the divalent metal ion into the cell.<sup>77,80</sup> In addition, small molecules may also be exported by cells to bind Mn(II) with high affinity and facilitate cellular uptake.<sup>81</sup> A molecule fulfilling such a nutritional role has yet to be definitively identified, however.

Bacterial Mn metabolism contributes to the global redox cycling of this element.<sup>82</sup> Mn(II) is readily soluble in water but more highly oxidized species typically deposit in the environment as insoluble Mn(III,IV) oxide minerals. Numerous microorganisms catalyse the oxidation of Mn(II), although whether this chemistry serves a biological function has yet to be definitively ascertained.<sup>83</sup> The enzyme-catalysed oxidation is proposed to proceed through sequential one-electron transfer steps, generating Mn(III) intermediates.<sup>84–86</sup> Indeed, such one-electron transfers are proposed to contribute to the persistent levels of soluble Mn(III) observed in suboxic zones.<sup>81</sup> The most widely studied manganese-oxidizing bacteria are fluorescent pseudomonads.<sup>87</sup> The luminescence of these microbes results from production of pyoverdine siderophores. These siderophores impact manganese metabolism, forming Mn-pyoverdine complexes. For instance, the Mn(II)-oxidizing strain *P. putida* MnB1 produces the pyoverdine PVD<sub>MnB1</sub>.<sup>88</sup> This molecule has all of the properties expected of a siderophore, but has a reported binding affinity for Mn(III) that is almost 1000-fold greater than that for Fe(III).<sup>88</sup> Mutant strains that overexpress pyoverdine demonstrate a reduced capacity to carry out Mn(II) to Mn(IV) oxidation.<sup>89</sup> Similar results were obtained using *P. putida* GB-1, which produces the pyoverdine PVD<sub>GB-1</sub>.<sup>87</sup>

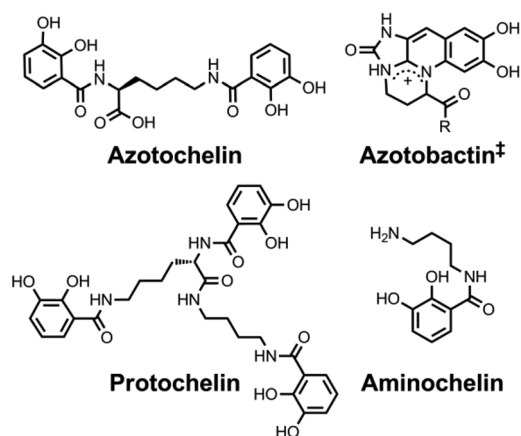
The investigation of siderophore–Mn(III) coordination chemistry was extended to other pyoverdines and rhizoferrin, and these molecules also coordinated Mn(III) with greater affinity than Fe(III).<sup>90</sup> It was proposed that the large siderophore–Mn(III) stability constants derive from the ability of these siderophores to accommodate the Jahn–Teller distortion of the high-spin  $d^4$  Mn(III) centre.<sup>90</sup>

We also briefly note here, in relation to biogeochemical cycling, that various hydroxamate and catecholate siderophores facilitate the dissolution of oxyhydroxide minerals containing Mn as well as Co, Cr, and Fe.<sup>91–93</sup>

### Fuelling nitrogenase: molybdenum and vanadium

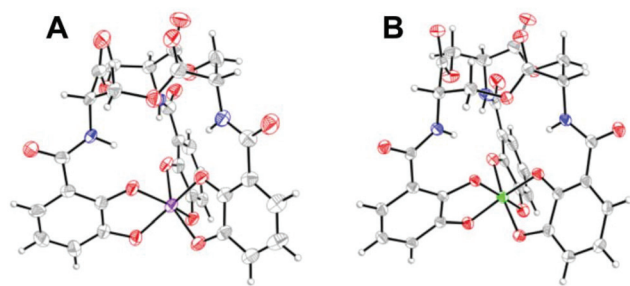
Nitrogenases are enzymes produced by diazotrophs that carry out the chemically challenging six-proton, six-electron reduction of dinitrogen to two equivalents of ammonia in water under ambient conditions.<sup>94</sup> The key to this transformation is an iron–sulphur-containing inorganic cofactor present within the enzyme. Whereas some bacterial strains produce nitrogenases with iron as the only metal in the cofactor, other strains incorporate molybdenum or vanadium into the cofactor as well.<sup>94–96</sup> Early studies identified aminochelin as a catecholamine siderophore produced by the nitrogen-fixing diazotroph *Azotobacter vinelandii* that coordinates both iron and molybdenum.<sup>97</sup> Using aminochelin, *A. vinelandii* was able to strip molybdenum from silicate samples.<sup>98</sup> Further studies on this model organism revealed that the siderophores it produces under Mo- and V-limiting conditions form stable complexes of these metals.<sup>99</sup> The biscatechol and triscatechol siderophores, protochelin and azotochelin, are secreted under these conditions and form 1 : 1 complexes with vanadate and molybdate as evidenced by tandem high performance liquid chromatography-inductively coupled plasma mass spectrometry.<sup>99,100</sup> Isotopically-enriched metal–siderophore complexes were used to confirm cellular uptake of the Mo- and V-bound forms of the metallophores. *A. vinelandii* also produces a pyoverdine-like siderophore called azotobactin that contains each of the three metal-binding motifs typical of siderophores: hydroxamate, catecholate, and  $\alpha$ -hydroxycarboxylate. Like protochelin and azotochelin, azotobactin coordinates vanadate and molybdate to form complexes that are transported into the bacterium.<sup>101</sup> Experiments using mutant strains deficient in the production of either azotobactin or the catecholate siderophores suggest that the latter are preferentially used to take up iron and the former, molybdenum.<sup>102</sup>

Although not related to an explicit biological function, we mention briefly here that, for many years, the vanadium complex of enterobactin was the closest analogue of the ferric complex of this archetypal siderophore to be characterized by



**Chart 3** Chemical structures of the siderophores discussed in *Fuelling Nitrogenase: Molybdenum and Vanadium*. †Azotobactin has a peptide chain as the R substituent.



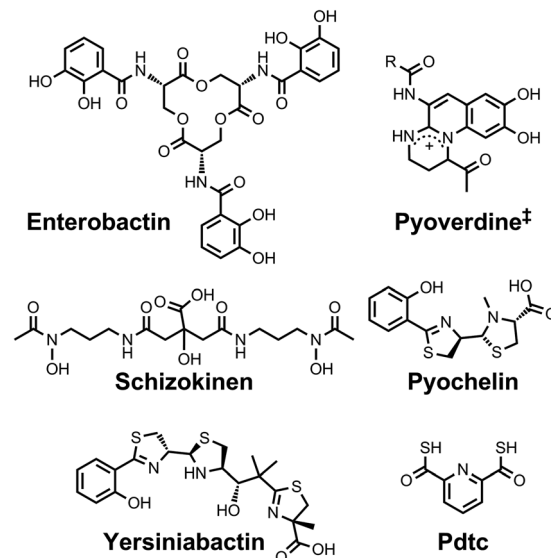


**Fig. 2** Thermal ellipsoid diagrams from the crystal structures of the (A) vanadium and (B) silicon complexes of enterobactin (CCDC ID: 624678 and 920703, respectively).<sup>103,105</sup> Thermal ellipsoids are drawn at the 50% probability level and hydrogen atoms are shown as spheres of arbitrary radius. Colour code: C gray, O red, N blue, V purple, and Si green.

atomic-resolution X-ray crystallography (Fig. 2).<sup>103,104</sup> Recently, structures of the silicon (Fig. 2), germanium, and titanium complexes of enterobactin were reported.<sup>105</sup> This work was motivated by the observation that enterobactin and salmocheilin S4 bind silicon.<sup>106</sup> Further insight into the structure of enterobactin can be obtained from crystal structures of ferric enterobactin complexed with different proteins. Early macromolecular structures of lipocalin-2 bound to enterobactin suggested that the trilactone was at least partially hydrolysed (Fig. 3).<sup>107,108</sup> A crystal structure of lipocalin-2 bound to what appears to be a non-hydrolysed ferric enterobactin complex was deposited in the protein databank in 2008 (PDB ID: 3CMP, Fig. 3), but no follow-up publication has appeared in the literature. More recently, a crystal structure was obtained of ferric enterobactin complexed with FeuA, a component of the trisaccholate siderophore transport machinery of *Bacillus subtilis* (Fig. 3).<sup>109</sup>

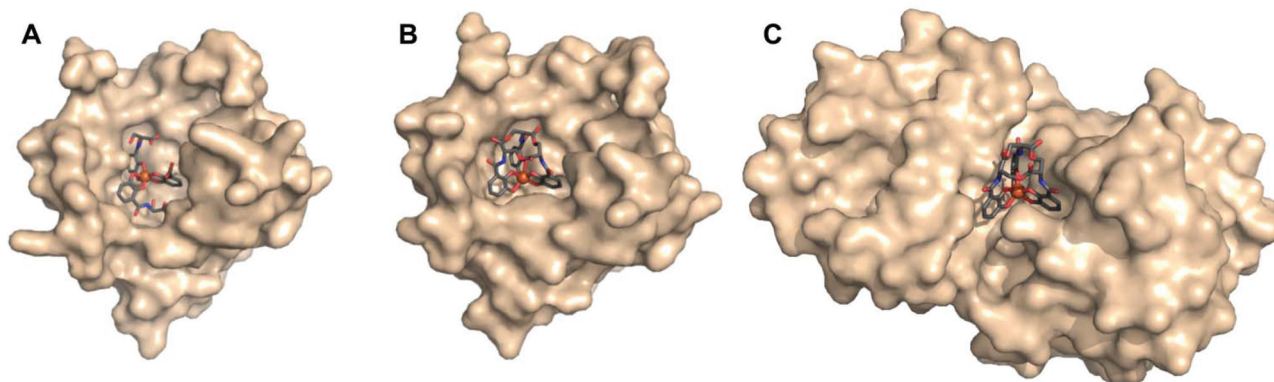
### The interaction of siderophores with copper

The concentration and localization of copper within cells is tightly regulated because of its propensity to catalyse deleterious Fenton-like chemistry.<sup>110</sup> Moreover, cellular over-accumu-



**Chart 4** Chemical structures of the siderophores discussed in *The Interaction of Siderophores with Copper*. The stereochemistry of yersiniabactin was taken from ref. 31. The enantiomer of the form of pyochelin shown, *enantio*-pyochelin, is a known siderophore. †Many pyoverdines exist and are distinguished by the nature of the peptide chain attached at the R position. Pdtc is pyridine-2,6-dithiocarboxylic acid.

lation of copper leads to toxicity.<sup>111</sup> Thus, bacterial copper transport proteins and chaperones are employed to control intracellular copper concentrations.<sup>112–116</sup> Although numerous studies have uncovered many aspects of the mechanisms by which bacteria use proteins to chaperone, sequester, and efflux copper, less is known about the means by which bacteria import copper.<sup>116</sup> Methanotrophic bacteria require particularly large supplies of copper to produce functional particulate methane monooxygenase.<sup>117,118</sup> This enzyme allows the bacteria to derive energy and the carbon they need for anabolic processes from methane.<sup>119</sup> Analysis of the growth medium in which methanotrophs were cultured under copper-limited conditions revealed the presence of small molecules that bind



**Fig. 3** Lipocalin-2 bound to either (A) hydrolyzed or (B) non-hydrolyzed ferric enterobactin (PDB ID: 3BY0 and 3CMP, respectively).<sup>108</sup> (C) Non-hydrolyzed ferric enterobactin complexed with FeuA (PDB ID: 2XUZ).<sup>109</sup> In all panels, the protein is shown as a wheat-coloured surface. The ligands of the protein-bound small molecule are shown as sticks and the iron atom as a sphere. Colour code: C grey, O red, N blue, and Fe orange.





copper with high affinity.<sup>120,121</sup> The compound identified in these studies that has received the most significant attention is called methanobactin.<sup>122,123</sup> This peptidic compound has been alternately referred to as a copper-binding ligand (CBL), a copper-binding compound (CBC), or a chalkophore. These names all serve to evoke an identical impression: this molecule binds copper ions and transports them into the cell.<sup>123</sup> Other molecules have been proposed to act as chalkophores, including coproporphyrin III, which is secreted by *Paracoccus denitrificans* under copper-limiting conditions to ensure an adequate supply of copper to the enzymes involved in denitrification.<sup>124</sup> The ubiquitous small molecule glutathione avidly binds copper but is thought to play a role in detoxification as opposed to transport.<sup>125</sup>

In addition to the small molecules described above, do siderophores bind copper? Similar to Zn(II), Cu(II) ions are softer than Fe(III) ions and so many of the siderophores that are well-tuned to complex ferric ions within a field of hard ligands will not interact as favourably with cupric ions. These two metal ions also prefer that their ligand fields assume different geometries, suggesting a lack of interaction between copper ions and siderophores pre-organized to bind Fe(III). Nonetheless, siderophores with softer donor atoms interact with copper. In the instances described below, this interaction is proposed have a specific biological purpose.

The pdtc metal-binding studies described above also identified copper as a metal that is coordinated by this siderophore.<sup>67</sup> A number of early studies on pdtc focused on the ability of its metal complexes to dehalogenate carbon tetrachloride,<sup>64,65</sup> and the cupric pdtc complex was identified as the most active species.<sup>126</sup> Although pdtc can act as a siderophore, it does not appear to be used by bacteria to increase intracellular copper concentrations.<sup>127</sup> The copper pdtc complex is capable of redox cycling, which may contribute to an as-yet undetermined biological function of this complex.<sup>67</sup>

Insight into the role that pseudomonal siderophores play in copper regulation was obtained by monitoring changes in the transcriptional profiles of *P. aeruginosa* following exposure to elevated copper concentrations.<sup>128</sup> These studies indicated that production of high-affinity pyoverdine siderophores increased under high-copper conditions, whereas levels of the low-affinity siderophore pyochelin decreased. These results were interpreted to indicate that pyochelin may be involved in the uptake of copper.<sup>128</sup> This proposal is in agreement with previous experiments, which revealed a decrease in pyochelin synthesis upon incubation of *P. aeruginosa* with copper as well as molybdenum, nickel, or cobalt.<sup>129</sup> Studies of the coordination chemistry of pyochelin confirmed that it forms stable complexes with Cu(II) and Zn(II).<sup>45</sup>

Some small molecules, such as glutathione, that interact with copper in bacterial cells do so to mitigate the toxicity of this element.<sup>130</sup> Copper, particularly Cu(I), is sufficiently toxic that mammals may use it as a bactericidal agent to combat microbial infection.<sup>131</sup> For example, cultured RAW264.7 mouse macrophage cells and peritoneal macrophages from freshly sacrificed mice appear to pump copper into phago-

somes containing *E. coli* in order to kill the bacteria.<sup>132,133</sup> Above, an immune response was described in which a host fends off an invading pathogen by starving it of essential nutrients. The pathogen can respond by secreting molecules, such as siderophores, to bind the nutrient metal ion with high affinity and increase its uptake. The situation is reversed with copper given that the microbe is bombarded with an excess of a nutrient, but surprisingly an increase in siderophore production again appears to be a viable survival strategy, as described below.

An investigation of the response of pathogenic bacteria to increased copper concentrations revealed that the microbes increase expression of efflux proteins and oxidases, and secrete molecules to sequester reactive copper species.<sup>130</sup> Glutathione and MymT, a bacterial metallothionein produced by *Mycobacterium tuberculosis* in response to high levels of copper, attenuate Cu(I) toxicity.<sup>113,114,125,134</sup> Moreover, molecules traditionally viewed as siderophores are employed by bacteria to grow under toxic concentrations of copper. In particular, yersiniabactin binds Cu(II) ions and prevents reduction to the more toxic Cu(I) oxidation state.<sup>135</sup> Conversely, catecholate-containing siderophores, such as enterobactin, reduce Cu(II), producing toxic cuprous ions.<sup>136</sup> As described below, the redox-active catechol moieties of enterobactin can alternatively play a cytoprotective antioxidant role.

Uropathogenic *E. coli* UTI89 delicately balance the desired iron-binding and undesired copper-reducing chemistry of catecholate siderophores. In the current model,<sup>135</sup> *E. coli* UTI89 produce yersiniabactin and enterobactin to sequester iron from host proteins. To combat infection, the host increases Cu(II) concentrations in the vicinity of the bacteria and these cupric ions react with the catecholate moieties of enterobactin to produce toxic cuprous ions. Increased production of yersiniabactin allows Cu(II) to be sequestered before it is reduced.<sup>135</sup> The cupric yersiniabactin complex is stable and has been detected in the urine of patients infected with uropathogenic *E. coli*.<sup>135</sup> It should be noted that expression of CueO, typically designated as a cuprous oxidase, can also be increased to mitigate the toxic effects of copper by oxidizing the enterobactin precursor 2,3-dihydroxybenzoic acid to 2-carboxymuconate, thereby preventing catechol-mediated reduction of Cu(II).<sup>136</sup>

The copper-yersiniabactin complex is competent to effect superoxide dismutation.<sup>133</sup> As noted above, expression of yersiniabactin confers a survival advantage upon pathogenic *E. coli* that are engulfed by macrophages. In addition to Cu(II) sequestration, the superoxide dismutase activity of copper-yersiniabactin may help to reduce the level of NADPH oxidase-derived superoxide that is produced by the macrophage to kill internalized bacteria.

Finally, we note that the ability of yersiniabactin and pyochelin to function in copper sequestration relies on the inability of the copper-siderophore complexes to interact with the receptors and transport proteins that mediate uptake of ferric siderophores. Nonetheless, experiments with the siderophore schizokinen indicate that this distinction is not univer-





sal. Production of this siderophore increases the sensitivity of *Bacillus megaterium* to copper by increasing intracellular Cu accumulation, but has the opposite effect in *Anabaena* spp.<sup>137,138</sup> In addition to copper transport by schizokinen, it is possible that alteration of iron uptake dynamics may factor into the ultimate toxicity observed in these experiments.

### Other metals: transport and sequestration

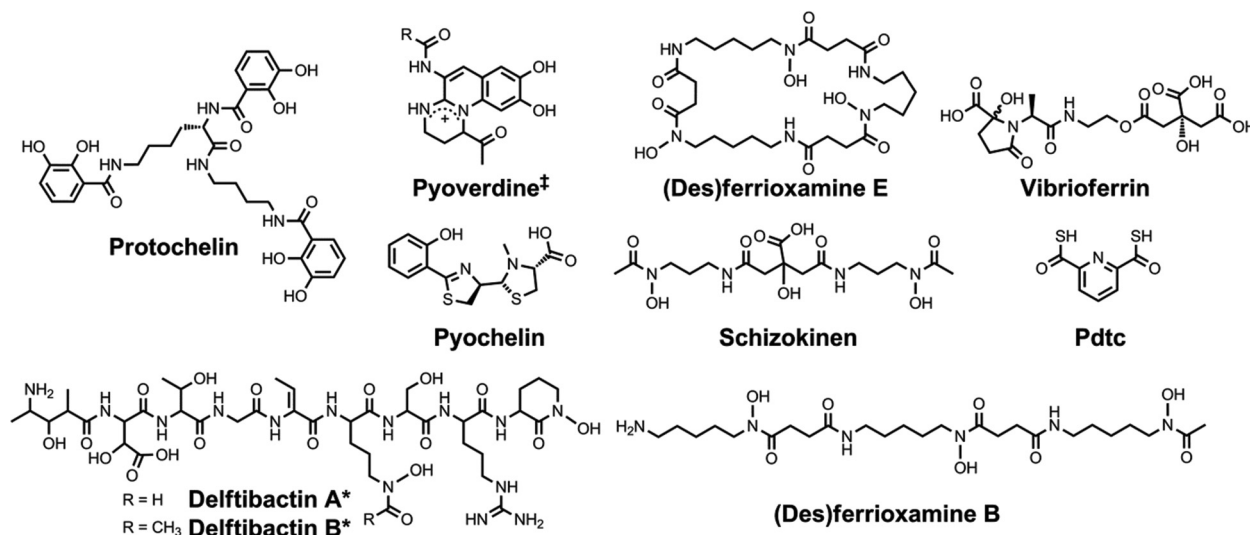
The coordination chemistry of a variety of siderophores has been studied using metals other than iron or those described above.<sup>67,139</sup> In addition to fundamental investigations of the abiological inorganic chemistry of some siderophore complexes,<sup>140</sup> a number of reports have focused on the broad-spectrum metal-binding capabilities of siderophores to probe the abilities of these ligands to mitigate or exacerbate the toxicity of heavy metals. The work described above on the protochelin-mediated transport of Fe, Mo, and V in *A. vinelandii*, which is important in evaluating the ability of this organism to satisfy the metal requirements of nitrogenases, was extended to include Cr and Co.<sup>141</sup> Protochelin is unlikely to play a role in the intracellular accumulation of Cr and Co, but interactions of these metals with protochelin have implications for Cr/Co biogeochemical cycling.<sup>141</sup>

The ability of the siderophores produced by *Pseudomonas* spp. to complex metals other than iron has received significant attention because of the prevalence of these bacteria in soil and marine environments as well as their classification as opportunistic human pathogens.<sup>145</sup> The pseudomonal siderophores pyochelin and pyoverdine form complexes with over 15 different transition metal and main group metal ions.<sup>146,147</sup> Moreover, these siderophore complexes were able to interact to varying degrees with their cognate receptors – the pyochelin complexes with FptA ( $K_d = 10$  nM–4.8  $\mu$ M) and the pyoverdine

complexes with FpvA ( $K_d = 2.9$  nM–13  $\mu$ M).<sup>148,149</sup> Despite the interaction that the non-iron pyochelin and pyoverdine complexes have with the appropriate outer membrane receptor, cellular accumulation was either absent or, in a few cases, present to a degree substantially less than that of the corresponding ferric complex.<sup>148</sup> Subsequent investigation revealed that the outer membrane transporters both recognize the non-iron metal complexes and import the complexes into the cell.<sup>149</sup> The lack of observed intracellular accumulation arises from the ATP-dependent efflux pump PvdRT-OpmQ, which exports pyoverdine complexes of unwanted metals. These properties have led to the investigation of *Pseudomonas* spp. as potential bioaugmentation agents in the phytoremediation of soils contaminated with environmental pollutants such as chromium, lead, and mercury.<sup>150,151</sup>

Pdte, the small thiocarboxylate-containing tridentate siderophore described above, was also interrogated for its ability to complex a wide range of metals and metalloids.<sup>67</sup> This ligand typically assumes a 1:2 metal-to-ligand ratio, affording an octahedral coordination sphere (Fig. 4). The ligand complexed 14 different metals and exerted a protective effect on *Pseudomonas* spp., *Staphylococcus* spp., an *Arthobacter* sp., *Bacillus* spp., *E. coli*, and *Candida albicans* that were incubated with mercury. Some of these organisms were also protected from cadmium and tellurium exposures.<sup>152</sup>

Although the work described above illustrates the ability of some siderophores to interact with a range of soft, heavy metals, it is not surprising that many siderophores interact better with smaller, harder metal ions. For instance, desferrioxamine B (DFOB) binds Fe(III), Al(III), Ga(III), and In(III) with log  $\beta_{110}$  values of 31.0, 24.5, 28.7, and 21.4, respectively.<sup>155</sup> This tight binding interaction is exploited to treat iron overload disorders, such as thalassemia, and aluminium overload



**Chart 5** Chemical structures of the siderophores discussed in *Other Metals: Transport and Sequestration and Marine Siderophores and Boron*. The enantiomer of the form of pyochelin shown, *enantio*-pyochelin, is a known siderophore. †Many pyoverdines exist and are distinguished by the nature of the peptide chain attached at the R position. \*The stereochemistry of the delftibactins has not been reported.<sup>142,143</sup> The chemical structure of vibrioferrin was obtained from ref. 144. Pdte is pyridine-2,6-dithiocarboxylic acid.



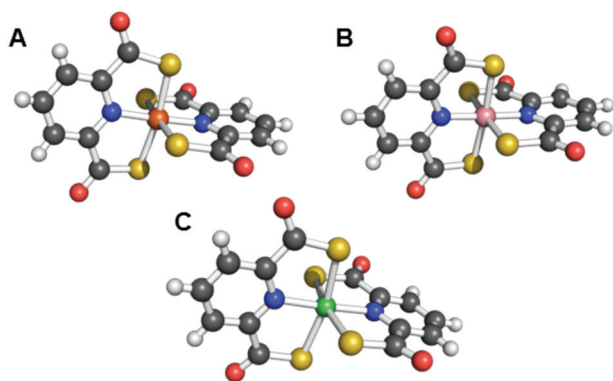


Fig. 4 Ball-and-stick representations of the metal complexes from the crystal structures of (A)  $[\text{Fe}(\text{pdte})_2]^{2-}$  (NBS ID: 557651),<sup>153</sup> (B)  $[\text{Co}(\text{pdte})_2]^{2-}$  (NBS ID: 596508),<sup>154</sup> and (C)  $[\text{Ni}(\text{pdte})_2]^{2-}$  (NBS ID: 596506).<sup>154</sup> Colour code: C gray, O red, N blue, H white, Fe orange, Co pink, and Ni green.

arising from ingestion of high levels of aluminium.<sup>156,157</sup> The siderophore schizokinen and its *N*-deoxy derivative, produced by *B. megaterium*, also bind aluminium.<sup>158</sup> The aluminium-siderophore complex was delivered into the cell through the siderophore transport receptor, leading to increased intracellular accumulation of aluminium.

In at least one instance, the strength of the interaction between a siderophore and aluminium resulted in the unintended complexation of the two.<sup>159,160</sup> Initial studies of the interaction of pyoverdine with its cognate outer membrane transporter FpvA appeared to indicate that the transporter bound the apo-siderophore with high affinity.<sup>161</sup> In depth investigation revealed that the strong transporter-small molecule interaction was in fact with the aluminium complex of pyoverdine, formed from trace amounts of aluminium in the buffers used for the experiments. The aluminium complex exhibits enhanced fluorescence as compared to the apo-siderophore,<sup>162</sup> which confounded the initial interpretation of the fluorescence resonance energy transfer data that were used to investigate pyoverdine-FpvA binding. We also note in passing that the first siderophore to be isolated, mycobactin (later renamed mycobactin P),<sup>163</sup> was initially isolated as an aluminium complex, the facile crystallization of which permitted detailed studies to be carried out on pure material.<sup>164–166</sup> The aluminium most likely originated from the alumina column that was used in the chromatographic preparation of the bacterial cell extract under investigation and is not involved in the biological function of mycobactin P.

$\text{Pu}(\text{iv})$  exhibits coordination chemistry similar to that of  $\text{Fe}(\text{iii})$ . The bioinorganic chemistry of plutonium is often studied with the aim of understanding its toxicity. Plutonium can bind transferrin and enter mammalian cells *via* the ferric transferrin uptake machinery.<sup>167</sup> The interplay between plutonium and microorganisms has also been investigated. Desferrioxamine (DFO) siderophores display high affinity for  $\text{Pu}(\text{iv})$ ,<sup>168</sup> for  $\text{Pu}(\text{iv})$ -DFOB,  $\log \beta_{110} = 30.8$  and  $\text{Pu}(\text{iv})$ -DFOE is sufficiently stable that its crystal structure was determined using X-ray diffraction methods (Fig. 5).<sup>169,170</sup> Studies with the

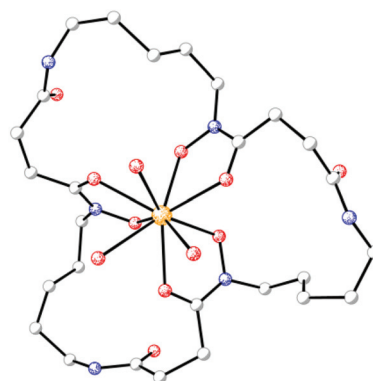


Fig. 5 Molecular diagram of the siderophore complex from the crystal structure of desferrioxamine E-triaqua-plutonium(IV) (CCDC ID: 136339).<sup>170</sup> Non-hydrogen atoms are shown as shaded spheres of arbitrary radius and hydrogen atoms have been removed for clarity. Colour code: C gray, O red, N blue, and Pu orange.

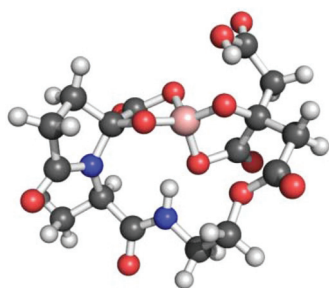
DFOB-producing bacterium *Microbacterium flavescens* JG-9 demonstrated that the  $\text{Pu}(\text{iv})$ -DFOB complex was taken up by living, metabolically-active bacteria.<sup>171</sup> As reviewed recently,<sup>172</sup> a number of subsequent studies have shown that, through the use of siderophores, microorganisms can dissolve plutonium and significantly affect its subsurface and environmental distribution.

The last example we will present in this section involves the delftibactins. These siderophore-like molecules are produced by the bacterium *Delftia acidovorans* and are products of NRPS machinery. The genes encoding the NRPS are clustered with genes of other siderophore synthases as well as siderophore transporters and regulators.<sup>142</sup> Like *Cupriavidus metallidurans*, *D. acidovorans* colonizes the surface of gold nuggets.<sup>173</sup> The delftibactins produced by *D. acidovorans* protect the bacterium from the toxic effects of gold and enable biomineralization of the metal as gold nanoparticles.<sup>142</sup> Enterobactin, yersiniabactin, and aerobactin were also tested for their ability to form gold nanoparticles.<sup>143</sup> Yersiniabactin and enterobactin were able to form small and large amounts of colloidal gold particles, respectively. In neither instance, however, were the particles formed as well or in a similar amount as with delftibactin A or B.<sup>143</sup>

### Marine siderophores and boron

The estuarine enteropathogen *Vibrio parahaemolyticus* is associated with episodes of gastroenteritis following consumption of contaminated seafood.<sup>174</sup> This bacterium produces vibrioferrin,<sup>175</sup> a citrate-based siderophore that is photoreactive and displays a relatively low affinity for  $\text{Fe}(\text{iii})$  ( $\log \beta_{110} = 24.0$ ) as compared to other marine siderophores.<sup>144,176</sup> Vibrioferrin was also identified in a search for siderophores in *Marinobacter* spp., and was unexpectedly observed as the borate complex (Fig. 6).<sup>177</sup> Given that boron was not added to the solutions from which the siderophore was isolated, the authors proposed that vibrioferrin was stripping boron from the borosilicate glassware used during the isolation procedure.





**Fig. 6** Ball-and-stick model of the proposed structure of the vibrioferrin-borate complex. The structure was minimized with *GaussView* based on that depicted in ref. 177. Color code: C gray, O red, N blue, H white, and B pink.

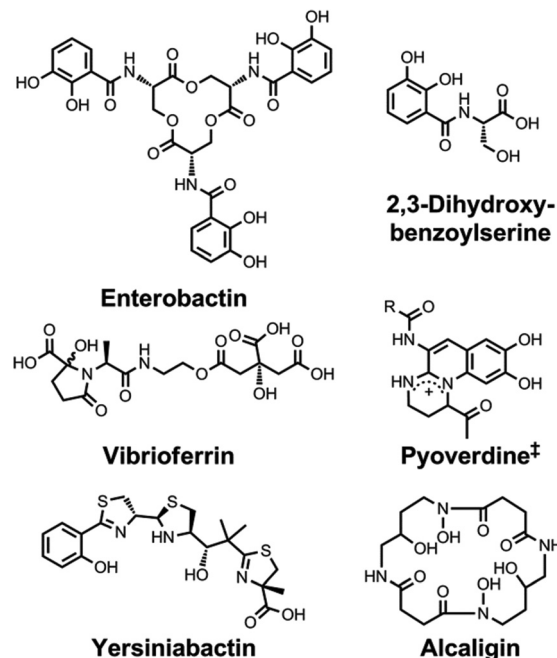
Further investigation revealed that a number of different citrate and catecholate siderophores form stable borate complexes.<sup>177</sup> Although borate is not typically present at very high concentrations in terrestrial environments, it is common in marine systems.<sup>178</sup> It is not yet clear whether the borate-siderophore complex has biological relevance; however, functions as either a boronophore or a signalling molecule have been proposed.<sup>177,179–181</sup>

Recent studies with *Marinobacter algicola* DG893 revealed that boron significantly influences the expression of at least 23 genes in this organism.<sup>182</sup> A number of the influenced genes are related to iron homeostasis. With the exception of FbpA, a periplasmic iron-binding protein, the expression of all of the iron uptake-related proteins examined increased with increasing boron concentration. The authors proposed that boron influences the iron uptake regulator, Fur.<sup>182</sup> Investigation into the interaction of borate with FbpA revealed that the protein synergistically binds Fe(III) and borate, effectively facilitating sequestration of the metal.<sup>183</sup> The mode of synergistic borate binding was proposed to be analogous to that of carbonate.<sup>184</sup>

We note that the *Marinobacter* genus was chosen as the subject of the aforementioned studies because it is frequently associated with toxic dinoflagellates such as *Gymnodinium catenatum* and *Scrippsiella trochoidea*. These algae are proposed to have a mutualistic relationship with *Marinobacter* whereby photolysis of ferric vibrioferrin releases iron to the benefit of the phytoplankton and the bacteria make use of the carbon fixed by the eukaryote.<sup>185</sup> This situation provides an example of a siderophore being used to feed an organism other than the producer to the benefit of the producer, in contrast to the well-established ability of bacteria to compete with one another by consuming xenosiderophores.<sup>186,187</sup>

### Siderophores as signalling molecules

The iron that is transported into cells by siderophores can act as a signalling agent, notably in the regulation of genes related to iron homeostasis.<sup>188</sup> It is also plausible that the siderophore itself, or a metal complex thereof, acts directly as a signalling molecule.<sup>189</sup> For example, the boron complex of vibrioferrin may act as either a traditional signalling molecule or a



**Chart 6** Chemical structures of the siderophores discussed in *Siderophores as Signalling Molecules*. The stereochemistry of yersiniabactin was taken from ref. 31. <sup>‡</sup>Many pyoverdines exist and are distinguished by the nature of the peptide chain attached at the R position. The chemical structure of vibrioferrin was obtained from ref. 144.

mediator of quorum sensing as described above.<sup>181</sup> The use of siderophores as signalling agents by *Pseudomonas* spp. is likely the most widely investigated manifestation of this chemical communication strategy.<sup>190–192</sup> The pyoverdines produced by *P. aeruginosa* strains serve not only to supply the bacteria with essential iron, and possibly carry out functions related to its binding to other metals as described above, but also regulate the expression of three virulence factors: exotoxin A, an endo-protease, and pyoverdines themselves.<sup>43,193</sup> Because the siderophore is secreted, it can signal within one bacterium as well as mediate communication between bacteria.<sup>194</sup> As a consequence of the regulatory effects that pyoverdines have on these virulence factors, mutants of the plant pathogen *Pseudomonas syringae* pv. *tabaci* 6605 deficient in their capacity to produce this siderophore exhibited reduced virulence in host tobacco infection.<sup>195</sup> Enhanced swarming ability and increased biosurfactant production were also phenotypic effects of the pyoverdine deficiency.

An early example of siderophores being used as signalling molecules came from the observation that some marine bacteria use exogenous siderophores to stimulate the production of endogenous siderophores.<sup>196</sup> For example, growth of the  $\alpha$ -proteobacterium strain V0210 was stimulated by the addition of *N,N'*-bis(2,3-dihydroxybenzoyl)-*O*-serylserine, a siderophore produced by *Vibrio* spp.<sup>196</sup>

Members of the *Bordetella* genus, including the pathogen *Bordetella pertussis* that causes whooping cough in humans, employ a range of alternative iron acquisition mechanisms to adapt to fluctuations in iron concentration during the course of



infection.<sup>197</sup> In *Bordetella* spp., the siderophores alcaligin and enterobactin function as inducers that activate expression of their cognate transport systems.<sup>198</sup> Because of this signalling capacity, these complexes have been referred to as *ferrimones*.<sup>198</sup>

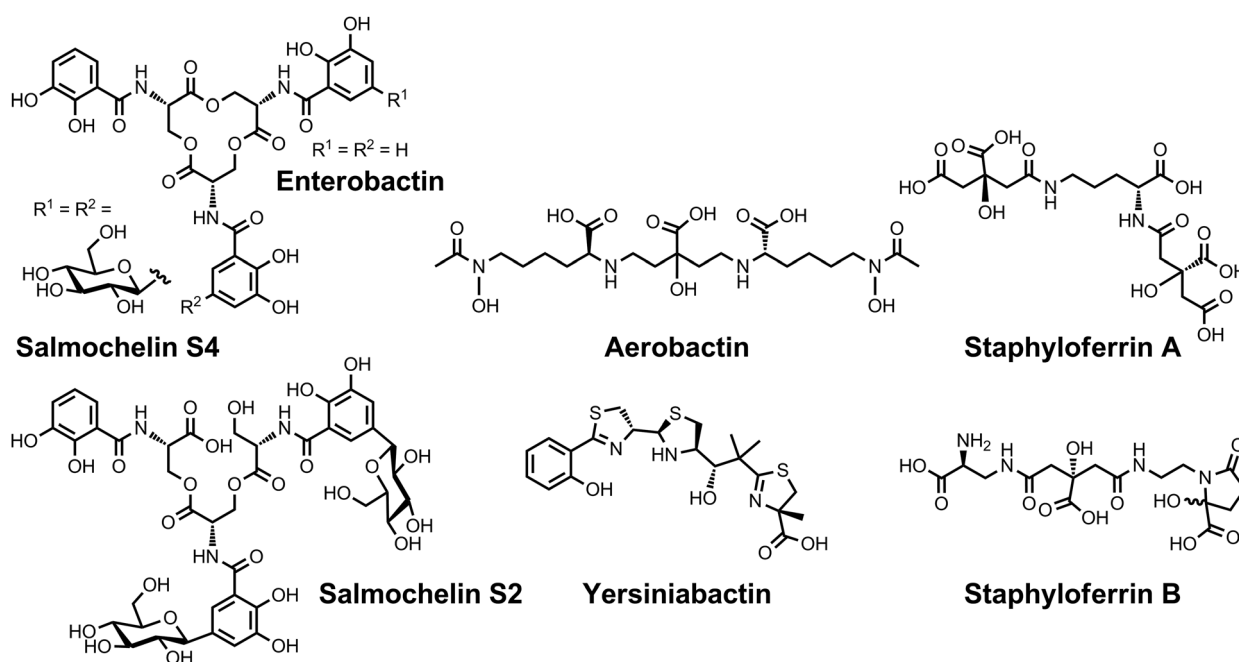
In *Y. pestis*, yersiniabactin can activate the transcription of the yersiniabactin outer membrane receptor (*psn*), the yersiniabactin synthetase HMWP2 (*irp2*), and a transcriptional regulator (*ybtP*). It also decreases expression of *ybtA*, another transcriptional regulator.<sup>199,200</sup> Importantly, yersiniabactin influenced transcription at concentrations 500-fold lower than those at which it exerted a nutritional effect, indicating that its signalling role functions independently of its nutritional role.<sup>199</sup> These genes, as well as yersiniabactin-mediated modulation of gene expression, were essential for *Y. pestis* to cause pneumonic and bubonic plagues in murine models.<sup>201</sup> We note that yersiniabactin-like molecules of unconfirmed structure produced by mutants with non-functional salicylate synthase YbtS or mutants that lack all *ybt* genes except for *ybtD*, a putative phosphopantetheinyl transferase, also modulated gene transcription in a similar manner.<sup>202</sup> Although these yersiniabactin analogues are not biologically relevant *per se*, such structure–activity relationship studies may provide insight into the nature of yersiniabactin signalling.

### Protection from oxidative stress

During an investigation of the antibiotic properties of pyochelin, enterobactin was observed to play a role in mitigating the deleterious effects of oxidative stress.<sup>203</sup> Addition of pyochelin

to *E. coli* increased levels of reactive oxygen species (ROS). The deleterious effects of these ROS were abrogated as a result of enterobactin production. Moreover, exogenous enterobactin supplementation provided protection to an *entE* mutant that could not biosynthesize enterobactin. Notably, this function is associated with the redox activity of the enterobactin catecholates rather than iron transport.<sup>204</sup> Moreover, *E. coli* strains that cannot produce enterobactin are unable to form colonies on minimal medium. This effect could not be reversed with iron supplementation; however, addition of enterobactin or a reducing agent such as ascorbic acid to the agar allowed the colonies to form, as did anaerobic incubation. On the basis of these results, the catecholate moieties of the siderophore are proposed to scavenge radicals.<sup>204</sup>

A similar siderophore-mediated protective effect was observed in *Salmonella enterica* serovar Typhimurium.<sup>207</sup> This enteric pathogen produces enterobactin and a series of glucosylated enterobactin derivatives known as salmochelins. The primary means by which these siderophores confer virulence is acquisition of iron from the host.<sup>42</sup> Recent studies indicate that these catecholate-based siderophores may also protect *Salmonella* from the oxidative stress encountered upon entering the macrophage. The enhanced survival of *Salmonella* producing salmochelins, as compared to mutant strains deficient in salmochelin biosynthesis and uptake, occurred primarily during the early stage of infection when the macrophage typically unleashes a cytotoxic oxidative burst. *In vitro* studies confirmed that catecholate siderophores, but not yersiniabactin or



**Chart 7** Chemical structures of the siderophores discussed in *Protection from Oxidative Stress*. The stereochemistry of yersiniabactin was taken from ref. 31. The chemical structures of staphyloferrin A and staphyloferrin B were obtained from ref. 205 and 206. The structure of salmochelin S2 is that reported in ref. 32. An alternative structure of a linearized salmochelin S4 in which a glucosyl unit is attached at the carboxylic acid end of the linearized trimer instead of the alcohol end has also been reported.<sup>33</sup> The stereochemistry of aerobactin is drawn so as to be consistent with the observation that only L-lysine is released upon degradation of this compound.<sup>245</sup>





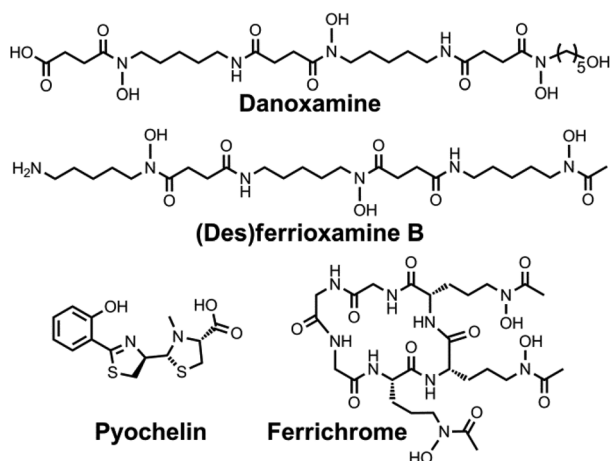
aerobactin, could protect *Salmonella* against oxidative stress and that the siderophore needed to be taken up into the cell to have this effect.<sup>207</sup>

The redox-mediated cytoprotective function of catecholate siderophores is reminiscent of the ability of cupric yersiniabactin to act as a superoxide dismutase and protect macrophage-internalized pathogens from oxidative stress.<sup>133</sup> As described above, however, results from the yersiniabactin studies put forward a role for the catecholate moieties of enterobactin in the generation of toxic cuprous ions that are used by the host organism to stem bacterial infections.<sup>135</sup> The combined results of these experiments indicate that catecholate siderophores can switch between cytoprotective and cytotoxic roles depending on the nature of the molecules in the environment surrounding the bacterium.

*Staphylococcus aureus* produces two citrate-based siderophores, staphyloferrin A and staphyloferrin B.<sup>208</sup> The SirABC protein system mediates uptake of the siderophore staphyloferrin B and contributes to virulence.<sup>209</sup> Under oxidative or nitrosative stress, *S. aureus* increases expression of *sirA*, as detected by qRT-PCR,<sup>210</sup> which conferred enhanced resistance to oxidative stress. It remains unclear whether this effect simply results from enhanced iron uptake or some other process.<sup>210</sup> Staphyloferrin B does not contain catecholate moieties,<sup>205,211,212</sup> so a direct analogy to the protective role that enterobactin plays in this regard is unwarranted.

### Sideromycins: siderophores as antibiotics

Sideromycins are a class of antibiotics in which the bactericidal warhead is attached to a siderophore.<sup>213</sup> The siderophore moiety performs targeting and delivery functions and thereby affords enhanced cellular uptake of the antibiotic for strains expressing the appropriate transport machinery. This “Trojan horse” drug delivery strategy has been employed in the design of a number of synthetic constructs aimed at enhancing therapeutic efficiency or overcoming antibiotic resistance.<sup>214–218</sup>



**Chart 8** Chemical structures of the siderophores discussed in *Sideromycins: Siderophores as Antibiotics*. The enantiomer of the form of pyochelin shown, *enantio*-pyochelin, is a known siderophore.

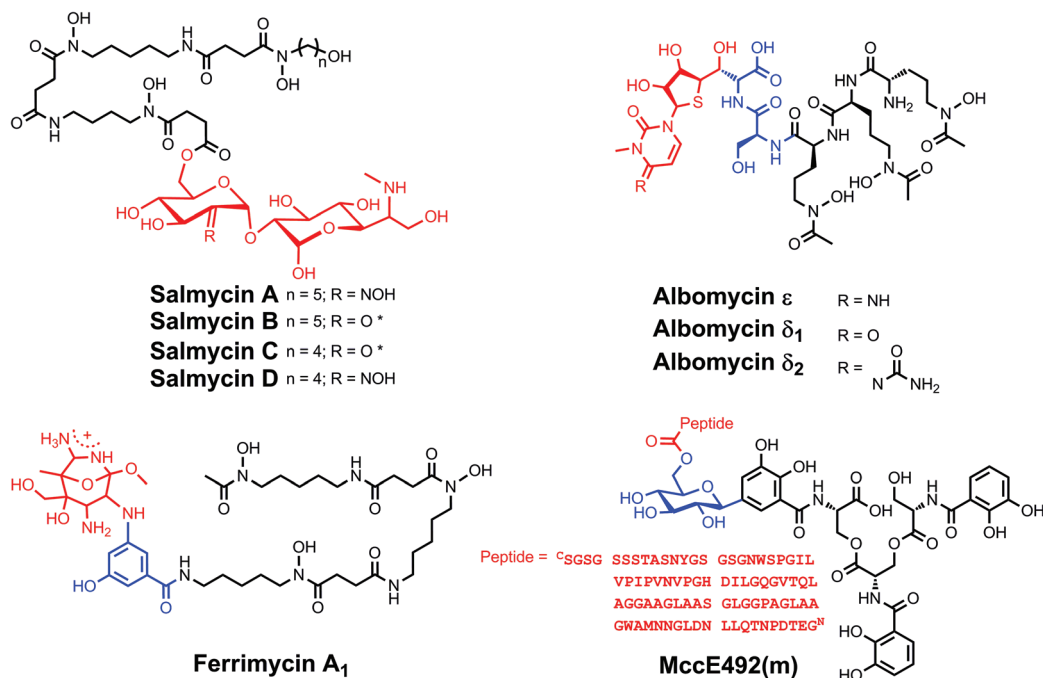
Examples include catecholate,<sup>219,220</sup> hydroxamate,<sup>221</sup> and carboxylate<sup>222</sup> siderophore conjugates. The significant, and growing, body of work on synthetic siderophore-antibiotic conjugates is a fertile ground for further investigation, and we refer the reader to the aforementioned recent reviews on the topic. Here, we will restrict our discussion to those siderophore-antibiotic conjugates that occur naturally.

Naturally occurring sideromycins were discovered before siderophores,<sup>223</sup> with the identification of a substance called *grisein* produced by *Streptomyces griseus*.<sup>224</sup> This compound is a member of a class of sideromycins called albomycins (Chart 9). These sideromycins feature a hydroxamate siderophore unit consisting of three *N*<sup>5</sup>-acetyl-*N*<sup>5</sup>-hydroxyornithine moieties, reminiscent of those found in fungal ferrichromes.<sup>20</sup> The C-terminus of the siderophore tripeptide motif harbours a serine that is linked to a nonproteinogenic  $\alpha$ -amino acid with a 4-thioxylfuranose side-chain. The modified furanose ring is bound to a cytosine, and modifications to the thioribosyl pyrimidine antibiotic group distinguish the different members of this family.<sup>225</sup> In all three albomycins, the pyrimidine is methylated at the *N*3 position. In albomycin  $\delta_1$ , the cytosine *N*4 is carbamoylated and in albomycin  $\epsilon$  it is not. In albomycin  $\delta_2$ , the *N*<sup>3</sup>-methylcytosine is replaced with *N*<sup>3</sup>-methyluracil.<sup>25</sup> These conjugates display a broad-spectrum of antibiotic activity against both Gram-negative and Gram-positive bacteria because of the widespread nature of transport proteins capable of binding ferrichrome-type siderophores.<sup>213,226</sup> The antibiotic activity stems from the ability of the pyrimidine unit to inhibit seryl-tRNA synthetase.<sup>227</sup> One intriguing feature of the albomycins is that the protein synthesis inhibitor is cleaved from the siderophore unit by intracellular peptidases to unleash antibiotic activity.<sup>228,229</sup> We briefly note that this feature of natural sideromycins can be used to inform the design of synthetic siderophore-antibiotic conjugates, in which a cleavable linker may be required.<sup>215,217,219</sup>

Salmycins (Chart 9), produced by a particular strain of *Streptomyces violaceus*, are another class of well-studied sideromycins.<sup>213</sup> They comprise a ferrioxamine siderophore unit conjugated to an aminodisaccharide.<sup>230</sup> Four salmycins, salmycins A–D, have been identified. In each case, the siderophore unit is danoxamine. Salmycins A and D are oximes of salmycins B and C, respectively. The aminodisaccharide unit is presumed to inhibit protein synthesis similarly to other aminoglycosides.<sup>174</sup> Danomycins A and B, the former of which is a carbamate of the latter, also feature a danoxamine iron-binding unit and an aminodisaccharide that inhibits protein synthesis,<sup>231</sup> but the detailed structures of these compounds remain unknown.<sup>232</sup>

Although three ferrimycins, *A*<sub>1</sub>, *A*<sub>2</sub>, and *B*, were isolated from *Streptomyces griseoflavus* ETH 9578, a comprehensive structural analysis appears to only be present for ferrimycin *A*<sub>1</sub> (Chart 9).<sup>213,223,232–234</sup> This compound features a DFOB siderophore unit linked to an iminoester-substituted lactam, *via* a 4-amino-5-hydroxybenzoate linker.<sup>26</sup> The heterobicyclic antibiotic warhead inhibits protein synthesis as demonstrated by studies monitoring the cellular incorporation of radiolabeled





**Chart 9** Natural sideromycins of known structure. The iron-binding siderophore unit is black, the antibiotic warhead is shown in red, and if a linker is present it is shown in blue. \*It is also possible that water is added such that this position bears a *gem*-diol.

phenylalanine by *S. aureus*.<sup>235</sup> In contrast to the albomycins, which exhibit broad-spectrum antimicrobial activity, the salmycins, danomycins, and ferrimycins are typically active only against Gram-positive bacteria.<sup>213</sup>

The final class of sideromycins that will be discussed is the siderophore-modified microcins. Microcins are small, ribosomally synthesized antimicrobial peptides that are produced by Gram-negative bacteria and used as narrow-spectrum antibiotics.<sup>236</sup> A number of microcins have been described, and different classification schemes have been put forth to categorize these molecules. A recently devised schema distinguishes between (i) class I microcins, which exhibit extensive backbone post-translational modification and are <5 kDa; (ii) class IIa microcins, which have no post-translational modifications except disulfide bonds and molecular weights in the 5–10 kDa range; and (iii) class IIb microcins that are linear polypeptides and may carry a C-terminal post-translational modification.<sup>237</sup> In this classification scheme, the class IIb microcins correspond to the catecholate microcins. These peptides are post-translationally modified with glucosylated catecholate siderophores, presumably to enhance cellular uptake by strains expressing catecholate siderophore receptors.<sup>237</sup> The class IIb microcins include MccE492, MccH47, MccI47, MccG492, and MccM. MccE492 was the first discovered to exhibit the catecholate siderophore modification, producing a form named MccE492m.<sup>238</sup> The post-translational modification is a linear trimer of 2,3-dihydroxybenzoylserine attached to the serine-84 carboxylate at the C-terminus of the peptide *via* a  $\beta$ -D-glucose linker (Chart 9). Effectively, the microcin is modified with a linearized and monoglucosylated enterobactin moiety.<sup>32</sup> Subsequently, biochemical studies confirmed that MccM and

MccH47, which were putatively assigned as siderophore-microcin conjugates based on bioinformatics analyses,<sup>239</sup> each contain C-terminal salmochelin-like post-translational modifications.<sup>240</sup> Chemical verification of the structures of MccG492 and MccI47 remains to be obtained.

Although MccE492 is potent in its unmodified form, with minimal inhibitory concentrations of 0.3  $\mu\text{M}$  in assays with various *E. coli* and *Salmonella* strains, the antimicrobial activity increases *ca.* 10-fold upon catecholate siderophore modification.<sup>238</sup> The increased activity of the modified microcin relies on the presence of siderophore uptake machin-

**Table 2** Biological functions of bacterial siderophores

Iron transport	The classical function of siderophores in which they are secreted to bind iron and transport it within bacteria to satisfy a nutritional iron requirement
Non-iron metal transport	Siderophores can bind other essential metals like Zn, Mo, V, and Mn to mediate their cellular uptake
Non-metal transport	Elements such as boron and silicon can bind siderophores and may be used by bacteria to take up these elements
Toxic metal sequestration	Heavy metals can be bound by siderophores to prevent their cellular entry
Protection from oxidative stress	Catecholate-bearing siderophores can scavenge harmful radicals and cupric yersiniabactin can function as a superoxide dismutase
Molecular signalling	Siderophores can regulate gene expression and potentially function in quorum sensing
Antibiotic activity	Siderophores can be conjugated to antibiotic warheads to increase efficacy



ery.<sup>238,241</sup> Although the class I microcin MccJ25 does not become post-translationally modified with a siderophore, it interacts with the ferrichrome receptor FhuA.<sup>242</sup> Recent X-ray crystallographic characterization of the complex of MccJ25 and FhuA revealed that the peptide mimics the binding of the siderophore to its receptor.<sup>243</sup>

## Conclusions and outlook

The study of siderophores has provided an opportunity for fundamental studies in coordination chemistry to make important contributions to understanding biological systems. Elucidation of the multifaceted role of a bacterial siderophore in metal homeostasis and bacterial physiology is a multidisciplinary endeavour that requires input from chemists, biochemists, and microbiologists. Approaching siderophores from a chemical standpoint is important for studies of non-classical siderophore functions. Undoubtedly, one of the most stunning attributes of siderophores is their ability to bind iron with remarkable affinity. Nevertheless, siderophores have a multitude of other chemical properties, and we now appreciate that bacteria will employ a siderophore for a secondary function provided that the siderophore can perform the requisite chemistry.

In the coming years, we expect that initiatives focused on the ability of siderophores to act as metallophores for other elements will continue on several fronts. Of particular interest is how this coordination chemistry contributes to metal-sequestering host-defence strategies and bacterial pathogenesis. Additionally, the influence of bacteria on the biogeochemical movement and environmental distribution of heavy metals *via* siderophore production is a rich area for continued investigation. Concerns over the fate of nuclear waste, particularly in the wake of natural disasters, will likely drive investigation into interactions of f-block elements with the siderophores of environmental microbes. With regards to sideromycins, many of the early discoveries of natural sideromycins require follow-up investigations. Such efforts have largely been eclipsed by initiatives to produce synthetic siderophore-drug conjugates to combat bacterial pathogens, including antibiotic-resistant strains. Although it lay outside the scope of this Perspective, we anticipate increased investigation into the use of siderophores as medicines to treat various human diseases. For instance, although DFOB is well-established for the treatment of iron-overload in conditions such as thalassemia and myelodysplastic syndrome, its use to treat other diseases, such as malaria, remains contentious.<sup>244</sup>

In addition to more focused investigations on the interactions of siderophores and siderophore-producing bacteria with non-iron metals, we expect that increased attention will be paid to larger mechanistic questions related to the means by which organisms employ siderophores for non-classical purposes without interference from the Fe-transport functions of these molecules. Also, better criteria will need to be established to delineate the difference between situations in which a siderophore simply exhibits the ability to interact with other

metals and those situations in which these interactions are biologically relevant.

In closing, the chemistry of siderophores is vast, even when restricted to iron transport. The wide expanse of chemical space representing the interactions of siderophores with non-iron elements is certain to contain a wealth of discoveries in chemistry and biology. We anticipate that a great increase in the number of non-classical functions attributed to these molecules is forthcoming as we come to better understand the subtleties of siderophore chemistry.

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