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MINIREVIEW

Multiple siderophores: bug or feature?

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Abstract
It is common for bacteria to produce chemically diverse sets of small Fe-binding molecules called siderophores. Studies of siderophore bioinorganic chemistry have firmly established the role of these molecules in Fe uptake and provided great insight into Fe complexation. However, we still do not fully understand why microbes make so many siderophores. In many cases, the release of small structural variants or siderophore fragments has been ignored, or considered as an inefficiency of siderophore biosynthesis. Yet, in natural settings, microbes live in complex consortia and it has become increasingly clear that the secondary metabolite repertoires of microbes reflect this dynamic environment. Multiple siderophore production may, therefore, provide a window into microbial life in the wild. This minireview focuses on three biochemical routes by which multiple siderophores can be released by the same organism—multiple biosynthetic gene clusters, fragment release, and precursor-directed biosynthesis—and highlights emergent themes related to each. We also emphasize the plurality of reasons for multiple siderophore production, which include enhanced iron uptake via synergistic siderophore use, microbial warfare and cooperation, and non-classical functions such as the use of siderophores to take up metals other than Fe.

Keywords Multiple siderophores · Secondary metabolites · Metallophores · Iron

Introduction

Siderophores, small Fe-binding molecules produced by microbes in response to Fe stress, are a well-studied class of secondary metabolites. While the role of siderophores in iron uptake is clear, the sheer diversity of siderophore structures is striking and the evolutionary advantages of siderophore diversity are not fully understood [1–4]. The production of variable small molecules that are functionally equivalent is not unique to siderophores (as reviewed by [1–4]). However, the (relatively) straightforward function of siderophores as Fe binders in combination with a growing body of literature regarding multiple siderophore production makes this topic worth revisiting. This review seeks to dissect reasons that might underlie the production of multiple siderophores.

Iron (Fe), an essential metal for microbial growth, is sparingly soluble in modern oxygenated environments. In response to this problem, many bacteria use siderophores to solubilize otherwise inaccessible Fe resources. Several basic features of siderophores are well established. Siderophores have higher affinities for Fe(III) than Fe(II) and other divalent cations. Siderophore production is often strongly regulated by Fe concentrations through the use of the Fur protein, which, when complexed to intracellular Fe(II), binds DNA regulatory elements, and, subsequently, leads to transcriptional repression of biosynthetic genes [5–7]. The siderophores discovered to date are composed of a limited set of Fe-binding moieties: hydroxamate, catecholate, and α-hydroxycarboxylate functionalities are the most common, but α-aminocarboxylate and α-hydroxyimidazole functionalities have been reported, as well. Metal coordination by siderophores has been treated in several other reviews [8–11] and is not discussed in detail here. Hundreds of siderophore structures have been characterized [10]. In many cases, their biosynthesis is well understood and occurs via non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS), or

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NRPS- and PKS-independent pathways [12–15]. Genome sequences are now available for thousands of microbes, and the presence of siderophore biosynthetic gene clusters can be identified in silico with relative ease. However, flexibility in the incorporation of siderophore precursors as well as the release of small fragments or precursors (Fig. 1) make it difficult to predict the full suite of siderophore structures produced by an organism without direct analysis.

Although the production of multiple siderophores has long been recognized, the mechanisms by which these variable products arise have not always been known or appreciated. As a result, multiple siderophore production (hereafter MSP) has been used to describe any instance in which more than one siderophore is isolated from an organism. Here, we make an effort to identify different routes to MSP and to point out emergent patterns related to each. We first introduce broad potential functions for the use of MSP and

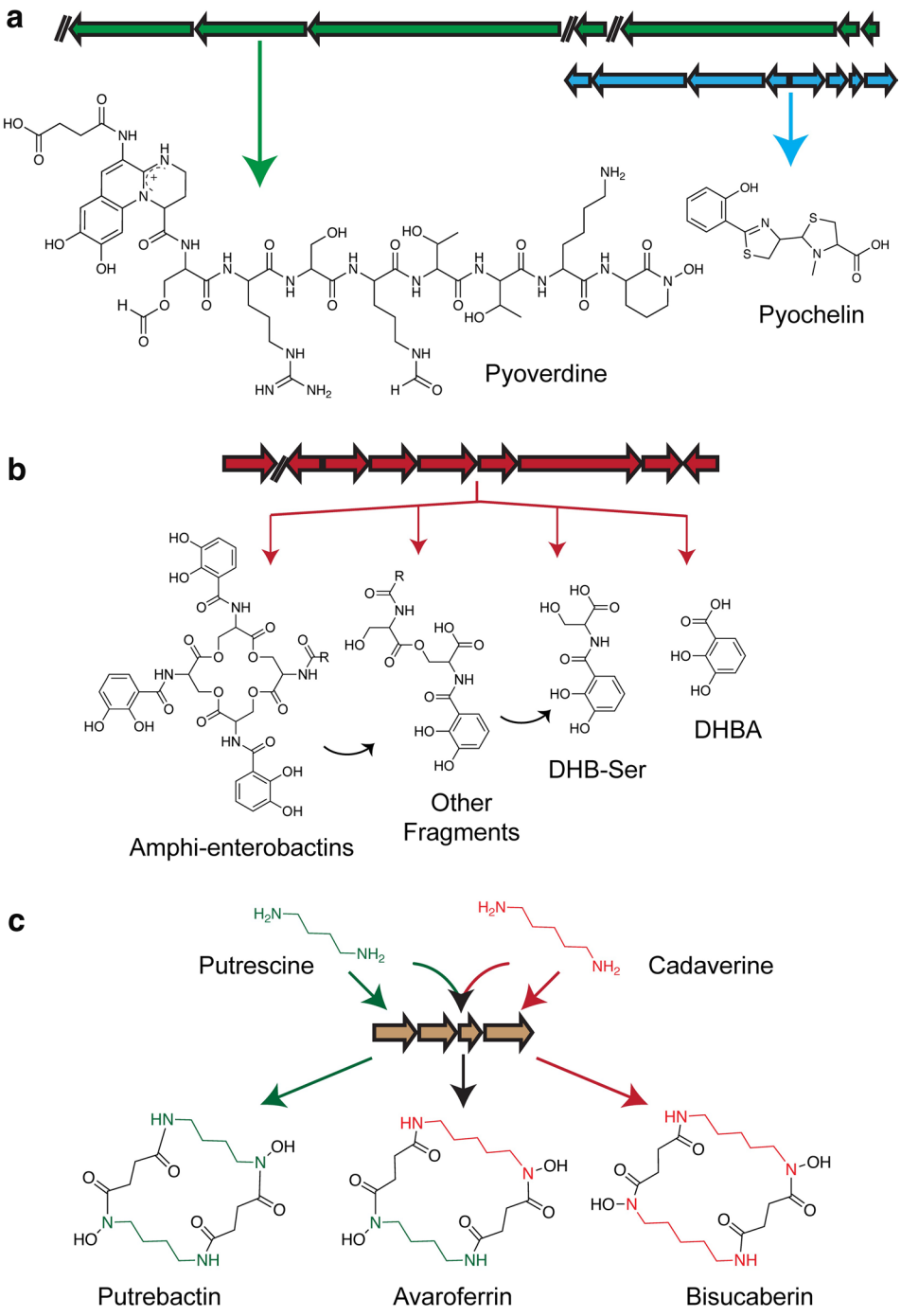

Fig. 1 Mechanisms for multiple siderophore production. **a** Multiple gene clusters. *Pseudomonas aeruginosa* encodes two separate siderophore gene clusters, which allow it to produce pyoverdine and pyochelin. **b** Release of fragments and precursors from the same gene cluster (red arrows) as well as potential degradation (black arrows). *Vibrio harveyi* encodes a gene cluster for the synthesis of amphi-enterobactins but releases numerous related fragments. **c** Incorporation of different precursors by the same gene cluster. *Shewanella algae* produces the siderophores putrebactin, avaroferrin, and bisucaberin in different amounts depending on the availability of the precursors putrescine and cadaverine

then discuss three principal processes by which it can occur (Fig. 1): (1) utilization of biosynthetically distinct gene clusters, (2) release of precursors and partial products, and (3) incorporation of variable precursors during biosynthesis. In the first scenario, organisms encode multiple biosynthetic gene clusters that produce structurally distinct siderophores. In the second scenario, an organism encodes genes for biosynthesis of a (typically large) siderophore, but smaller fragments of the full product accumulate during growth. In the last scenario, changes in intracellular metabolite pools dictate the incorporation of different siderophore precursors into the final product. We also note that multiple siderophores can often be produced through abiotic photochemical reactions, but do not discuss this topic as it has been reviewed elsewhere [9].

An overarching premise of this minireview is that the presence of multiple siderophore biosynthetic capacities within a genome conveys useful information about siderophore functions—both through the pairings of different types of siderophores and the selection of biosynthetic enzymes that may allow for more or less diversity in the siderophores that are generated. Studying MSP, therefore, provides insights into the important but complex topic of environmental siderophore function. Our goal in this minireview is to harness the existing knowledge regarding structures, biosynthesis, and metal coordination to begin to identify broad trends in multiple siderophore production. We note that this discussion requires knowledge of the entire suite of siderophores produced by an organism as well as the quantities in which these siderophores are synthesized, information that is available in a few cases. Accordingly, this minireview also functions as a call for more holistic studies that quantify all siderophores synthesized by an organism. A wealth of information is currently available regarding siderophore structures and modes of Fe chelation; applying this knowledge to microbial siderophore use in the environment is now feasible and represents an important new research challenge.

Functions of MSP: microbial interactions, iron storage, and metallophores

While siderophores are clearly used in Fe uptake, the advantage of multiple siderophores remains unclear. One common explanation for MSP is that it serves as a strategy for microbial competition or cooperation. Indeed, 'siderophore piracy' or the use of siderophores synthesized by other organisms has been well documented. Organisms frequently encode transporters for siderophores that they do not themselves synthesize and microbes from a wide variety of environments have been shown to utilize exogenous siderophores [16–23]. Notably, in some studies, siderophores from co-occurring organisms are assumed to be xenosiderophores without the further verification of siderophore biosynthetic capacity of the experimental organism. Recently, co-culture studies have given detailed insight into siderophore exchanges between microbes and provide compelling evidence for siderophore piracy. Traxler et al. [24] showed that the streptomycete *Amycolatopsis* sp. AA4 downregulates siderophore biosynthetic genes in co-culture with the desferrioxamine E (DFO-E) producer *Streptomyces coelicolor.* They also demonstrated that addition of DFO-E restores Fe-replete phenotypes in *Amycolatopsis* sp. *AA4*, suggesting that this organism trades endogenous for exogenous siderophores when they are available. Galet et al. [25] reported similar results in a different system: co-culture studies demonstrate that *Pseudomonas fluorescens* strain BBc6R8 downregulates pyoverdine and enantiopyochelin production when grown in the presence of *Streptomyces ambofaciens* ATCC 23877. *P. fluorescens* also upregulates the TonB-dependent receptor *foxA* in the presence of *S. ambofaciens.* The authors concluded that *foxA* is a siderophore transporter that allows *P. fluorescens* to utilize *S. ambofaciens* ATCC 23877 siderophores.

Given the well-documented possibility of siderophore piracy, MSP is thought to increase the chances that at least one siderophore remains inaccessible to competitors. The importance of siderophore diversity in this context is, perhaps, best illustrated by pathogenic organisms, which must compete not only with other microbes but also with host immune defenses. Pathogenic *Escherichia coli* and *Salmonella enterica* produce salmochelins—glycosylated and linearized versions of the siderophore enterobactin. While enterobactin is captured by the mammalian protein siderocalin, salmochelins are not [26–28]. Salmochelins are produced by tailoring enzymes that modify the enterobactin core. The use of biosynthetically distinct siderophores, or small variants derived from the incorporation or release of different precursors (Fig. 1) should presumably provide similar benefits. Rather than acting as a simple defense against siderophore theft, MSP can instead be used as an offensive strategy to monopolize Fe and starve competitors. Exogenous siderophores have, in fact, been shown to halt microbial swarming motility, development, and biofilm formation [24, 29–31]. Siderophores are also involved in cooperative microbial interactions, such as those between marine bacteria and eukaryotic phytoplankton [32]. The addition of exogenous siderophores has also been shown to stimulate microbial growth [16, 17, 19, 20]. Interestingly, there seems to be no well-studied example of microbes from different species co-producing and sharing siderophores. However, exogenous siderophores have been shown to stimulate siderophore production [33]; whether this is competitive or cooperative remains unknown.

There is also growing evidence that siderophores are used for purposes other than extracellular Fe acquisition and

sequestration [34]. Siderophores have cell-signaling as well as antibiotic properties [34], the latter often being related to the production of reactive oxygen species [35]. In addition, many fungi lack the iron storage protein ferritin and some species use siderophores for this purpose, producing one type of siderophore for extracellular iron acquisition and another for intracellular storage. The presence of intracellular siderophores was first documented in *Neurospora crassa* [36], but has since been found in other fungi. For example, *Aspergillus* sp. produce two sets of structurally similar hydroxamate siderophores: fusarins, which are excreted, and ferricrocins, which are kept intracellularly [37, 38]. A similar pattern (with slightly different siderophore variants) is seen in *Penicillium chrysogenum* [37]. The use of intracellular siderophores appears to be widespread among fungi [10, 39, 40] and may explain the presence of MSP in many species.

In addition, some siderophores may actually be metallophores used in the uptake of trace metals besides iron [34, 41–43]. This concept can be confusing (most siderophores will exhibit some capacity to complex other metals) and necessitates a more explicit definition that conveys the 'intention' of metallophore production. Kraemer et al. [42] adopted a particularly stringent definition for metallophores, proposing that (1) they must be used by the organism to obtain the metal nutrient of interest and (2) their production must be regulated by the organism's nutritional status in regard to the metal. Recently, several examples that meet these criteria have been established. Methanotrophs produce copper-binding chalkophores called methanobactins which are up-regulated under copper limitation and are used to meet the copper demands of the methane-monooxygenase enzyme [44, 45]. The production of catecholate molybdophores and vanadophores to take up the nitrogenase co-factors Mo and V has also been shown in the model nitrogen-fixing bacterium *Azotobacter vinelandii*. The Mo–protochelin and V–protochelin complexes are selectively taken up by Mo- and V-limited cells to meet metal demands [46–48] and the production of protochelin is also increased under Mo—(but interestingly not V)—limitation [49]. Based on these examples, the use of metallophores may be more widespread than currently realized and could offer an explanation for MSP: some siderophores may be used solely for Fe uptake, while others are used also or exclusively for the uptake of other metals.

Multiple siderophore gene clusters in the same organism: siderophores with high and low Fe affinities as an emergent theme?

In addition to the role of MSP in microbial interactions, biosynthetically distinct sets of siderophores could also work in concert to provide Fe to the cell. The use of 'synergistic' siderophores [4] might be manifest in microbial biosynthetic repertoires that consistently pair siderophores with complementary properties. One established example is the 'bucket brigade' mechanism, whereby more hydrophilic siderophores scavenge Fe and shuttle it to more hydrophobic siderophores [50–52]. Shuttling of Fe from hydrophilic to hydrophobic siderophore variants has been shown in *Mycobacterium tuberculosis,* and some *Mycobacteria* species are known to make biosynthetically distinct siderophores with different hydrophobicities [53, 54]. This process has also been proposed as an explanation for the production of amphiphilic siderophores with hydrophobic tails of different lengths (further details on variation in amphiphilic siderophore membrane partition coefficients are reported by [50, 55]).

Another emerging example of siderophore synergy is the production of siderophores with high and low Fe(III) affinities. The advantages of this pairing have been predicted by abiotic studies showing that a combination of siderophores and oxalate enhances Fe dissolution. However, examinations of MSP show that many bacteria do, in fact, produce siderophores with both high and low Fe affinities. Several studies have reported that rates of Fe dissolution from the iron oxide goethite are higher in the presence of the weak organic ligand oxalate and the hydroxamate siderophore desferrioxamine B (DFO-B) than in the presence of either alone [56, 57]. The general mechanism proposed is the adsorption of oxalate at mineral surfaces, the formation and subsequent detachment of the labile Fe–oxalate complex, and ligand exchange reactions between oxalate and DFO-B, which leave oxalate free to react with goethite again (Fig. 2a). A particularly notable result from these studies is that high concentrations of oxalate decrease the concentration of DFO-B needed to obtain a given Fe-dissolution rate—thus theoretically maximizing the utility of siderophore production.

Pairings of siderophores in several species suggest that organisms have found ways to take advantage of this Fe-dissolution mechanism (Fig. 2a–c). *Pseudomonas aeruginosa* PAO1 synthesizes both pyoverdine (**1**) [58], a siderophore with a high affinity for Fe (pFe = 27, [59]) and pyochelin (**2**) [60], which has a lower affinity for Fe (pFe = 16, [61]). *Burkholderia cepacia* ATCC25416 makes the hydroxamate siderophore ornibactin (**3**) [62] as well as

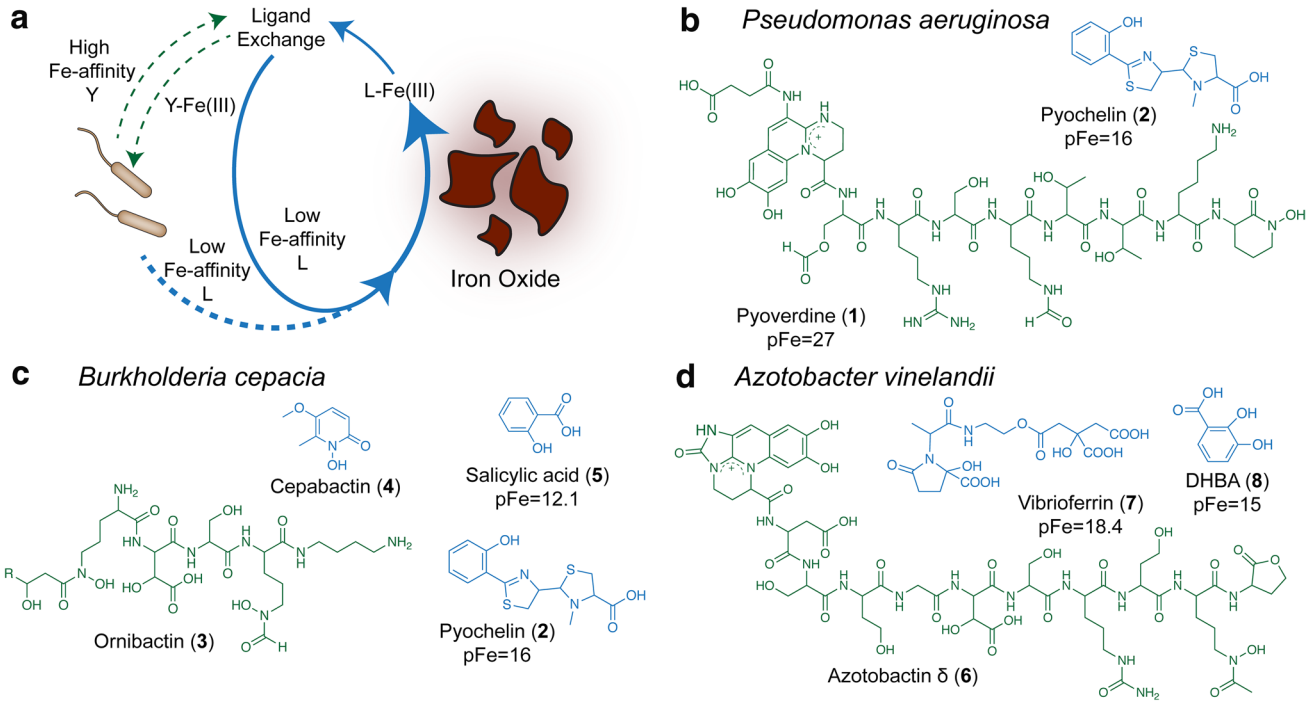

Fig. 2 Synergistic use of siderophores with high and low Fe(III) affinities. **a** Cartoon of high Fe-affinity (Y) and low Fe-affinity (L) siderophore interactions. Both types of siderophores are produced by the same organism. Siderophores with low affinities for Fe(III) form labile Fe(III) complexes: L–Fe(III). Fe(III) is exchanged with the high-affinity siderophore (Y), forming Y-Fe(III) which is taken up by cells to meet Fe demands. This leaves L free to react with iron again, as proposed by [56, 57]. Microbial siderophore release and uptake are depicted with dashed lines. High (green) and low (blue) Fe(III)-affinity siderophores produced by **b** *Pseudomonas aeruginosa*, **c** *Burkholderia cepacia,* and **d** *Azotobacter vinelandii*. pFe values (defined as –log $[Fe^{3+}]$ at pH 7.4, with $[Fe^{3+}]_{total}=1$ μM, $[Siderophore]_{total}=10$ μM) are taken from: [74], DHBA; [61], pyochelin; [59], pyoverdine; [65], salicylic acid; [69], vibrioferrin

pyochelin (**2**) and cepabactin (**4**) [63]. While pFe values for ornibactin have not been reported, those available for other hexadentate hydroxamate siderophores range from 23.3 (aerobactin) to 27.7 (ferrioxamine) (hydroxamate pFe values are compiled in [64]). Salicylic acid (**5**), a precursor of pyochelin with a low Fe affinity (pFe = 12.1, [65]) has also been found in *Burkholderia* supernatants [66]. *Azotobacter vinelandii* synthesizes azotobactin (**6**) [67, 68], which is structurally similar to pyoverdine (pyoverdine pFe = 27, [59]) as well as the α-hydroxycarboxylate siderophore vibrioferrin (**7,** pFe = 18.4, [69]) [70]. Interestingly, *A. vinelandii* also makes a suite of biosynthetically related catechol siderophores [71–73], which include 2,3-dihydroxy-benzoic acid (DHBA, **8,** pFe = 15, [74]) as well as the mono-catechol aminochelin (**9,** pFe = 17.6, [75]), bis-catechol azotochelin (**10,** pFe = 23.1 [71]), and triscatechol protochelin (**11,** pFe = 27.5 [71]) (see discussion of fragment production below). A related species, *Azotobacter chroococcum* ATCC 4412, also has a gene cluster for the biosynthesis of vibrioferrin and has been recently shown to produce chrochelins, siderophores with a higher Fe affinity (pFe = 23.9 for chrochelin vs. 18.4 for vibrioferrin [69]).

In *A. vinelandii*, the concentrations at which these siderophores are released fit expectations from abiotic studies; siderophores with lower Fe affinities are produced at much higher concentrations than those with higher affinities. This pattern is also seen in *V. harveyi*, although the mechanism for siderophore production is different (see discussion of siderophore fragments). Vibrioferrin is one of the most abundant siderophores synthesized by *A. vinelandii* and is produced at ~ 10× the concentrations of the catechol siderophores and azotobactin [49, 70]. Accordingly, in *A. vinelandii*, azotobactin production only occurs under extreme Fe limitation [41, 70], and when synthesized, this siderophore is not typically produced at high concentrations [70]. *A. vinelandii* could condition its environment through the release of high concentrations of vibrioferrin followed by much lower concentrations of siderophores such as protochelin (or under extreme Fe limitation, azotobactin), which have higher Fe affinities. It is also possible that *A. vinelandii* uses a bucket brigade mechanism, since vibrioferrin is very hydrophilic and may scavenge Fe from the external milieu and shuttle it to more hydrophobic catechols [70].

Iron limits microbial growth in many environments and it is, perhaps, not surprising that microbes should utilize

siderophore pairings to optimize Fe bioavailability. Siderophore biosynthetic gene clusters are often transferred horizontally. However, the utility of siderophores with high and low pFe values or hydrophobic and hydrophilic pairings may lead to the retention of specific biosynthetic complements. The production of siderophores with varying Fe affinities as well as hydrophobicities can also be achieved via fragment release and precursor-directed biosynthesis (Fig. 1b, c), as discussed below. However, the use of multiple biosynthetic gene clusters is distinct from other mechanisms of MSP as it creates the opportunity for differential regulation at the transcriptional level. Organisms could theoretically, and in some cases, do, produce large amounts of siderophores with low Fe affinities and only small amounts of siderophores with higher Fe affinities. Studies reporting the concentrations of multiple siderophores are rare, making it difficult to assess whether this general pattern holds across organisms. In *P. aeruginosa*, for example, higher Fe-affinity siderophores actually seem to be produced in greater concentrations than lower Fe-affinity siderophores [58, 60, 76–78], although siderophore production in this organism is complex [79–81]. As we gain more information about the types of siderophores made by different organisms and the concentrations in which these molecules are synthesized under a variety of environmental conditions, more broad trends may emerge.

Release of siderophore fragments

Many organisms appear to accumulate 'fragments' or 'incomplete' versions of siderophores in the growth medium. Due to their typically small size and (relatively) low affinity for Fe(III), these molecules are often considered to be byproducts of the larger siderophore. However, several studies show that fragments and precursors are produced in high concentrations and contribute to Fe uptake. One of the best examples of fragment release comes from catechol siderophore biosynthesis where the release of the siderophore precursor 2,3-dihydroxy-benzoic acid (DHBA) as well as other catechol monomers occurs in a number of organisms. DHBA accumulates in the culture medium of organisms that produce the triscatechols protochelin [72] and amphi-enterobactin (**13–16**) [82]. Many catechol producers also accumulate other, more complex mono- and bis-catechol variants. Organisms that produce the triscatechols cyclic-trichrysobactin, trivanchrobactin, turnerbactin, and (amphi-)enterobactin release the catechol monomers chrysobactin [83, 84], vanchrobactin [85], DHB-Orn-Ser [86], and DHB-Ser (**12**) [82, 87], respectively. In addition to the triscatechol protochelin, *A. vinelandii* releases three other variants: the bis-catechol azotochelin [72] ,and the monocatechols DHBA and aminochelin [71].

The accumulation of these fragments could result from hydrolysis (biotic or abiotic), precursor leakage from cells, early release of fragments from the assembly line, or some combination of the three. Base-catalyzed abiotic hydrolysis of enterobactin has been documented [88]. Enzymatic hydrolysis also occurs during the uptake of the enterobactin–Fe complex, which must be hydrolyzed to release iron [89–93]. Nonetheless, siderophore fragments are often found in extremely high concentrations, suggesting that early release from the assembly line or leakage from cells may be occurring. For example, in *A. vinelandii,* it is clear that mono- and bis-catechols are released from the NRPS assembly line [70]. The potential biosynthetic mechanisms by which other catechol fragments might be released from biosynthetic assembly lines have recently been reviewed by Reitz et al. [94]. Single amino acid changes in in vitro studies of the enterobactin NRPS interrupt oligomerization and promote the leakage of monomers through hydrolysis. There is evidence for protective amino acids that, when mutated, allow for further hydrolysis and reduced cyclization. However, sequences of the NRPS biosynthetic genes from several organisms that seem to release monomers do not always have mutations in these amino acids [94].

In the case of DHBA, which is synthesized from chorismate, the monomer could be released from cells prior to incorporation into the larger siderophore [70, 95] (Fig. 3). DHBA is infrequently measured in siderophore studies, but catechol biosynthesis is a well-conserved pathway and DHBA release could be widespread. For example, enterobactin [13, 96], cyclic-trichrysobactin [94], trivanchrobactin

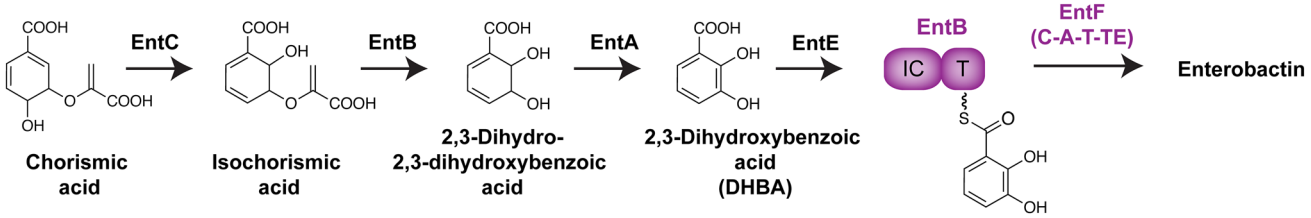

Fig. 3 DHBA synthesis from chorismate. DHBA is synthesized from chorismate before incorporation into the NRPS assembly line. The pathway for enterobactin production is shown, but analogous DHBA biosynthesis exists in many organisms. A: adenylation domain; C: condensation domain; IC: isochorismatase; T: thiolation domain; Te: thioesterase domain

[94], and turnerbactin [86] utilize similar biosynthetic pathways (see [94] for further examples). An analogous phenomenon occurs in organisms that synthesize salicylic acid-containing siderophores. Like DHBA, salicylic acid is often produced from chorismate before being incorporated into the NRPS assembly line. *B. cepacia* releases the siderophore pyochelin as well as its precursor salicylic acid [66]. Species of *Mycobacteria* that synthesize mycobactins (which incorporate salicylic acid) also release this precursor into the growth medium [97, 98]. Notably, salicylic acid acts as a signaling molecule especially in plant–microbe systems [99], and its release may have multiple functions.

Due to their relatively low Fe affinities, DHBA and other monomers are often ignored in siderophore studies. Triscatechol siderophores form much stronger complexes with Fe than mono- and bis-catechols, and are, therefore, usually assumed to be the most important siderophores. However, in several cases, monomers are found in high concentrations and have been shown to enhance Fe uptake. In *A. vinelandii*, the sum of the mono- and bis-catechols DHBA, aminochelin, and azotochelin concentrations is comparable to that of the triscatechol protochelin [49, 70, 100] (Fig. 4a). We recently reported that, in *V. harveyi,* DHBA and DHB-Ser accumulate to much higher concentrations than amphi-enterobactin (Fig. 4b, [82]). Radio-tracer (^{55}Fe) experiments also show that the addition of DHBA as well as spent medium (containing DHBA and DHB-Ser) leads to substantial increases in Fe uptake in *V. harveyi* [82]. This finding is consistent with the studies of enterobactin biosynthesis in *E. coli*, which used radio-tracers to show that DHBA and DHB-Ser enhance Fe uptake in this organism [101, 102].

Catechol moieties are one of the most common siderophore functionalities and their biosynthesis is well conserved across a wide taxonomic and environmental range of organisms. The accumulation of DHBA and other siderophore monomers may be a general feature of this type of siderophore biosynthesis. Further studies are needed to verify whether hydrolysis, early release, and/or leakage are responsible for their presence and to determine the contributions of fragments to Fe uptake. However, regardless of the route by which fragments are produced, if their production is in fact widespread and follows the models of *V. harveyi* and *E. coli* (where fragments make significant contributions to Fe uptake), it suggests that a number of organisms currently assumed to rely on triscatechol siderophores are, in fact, also utilizing a variety of bis- and mono-catechols. The release of siderophore precursors typically results in the simultaneous production of siderophores with relatively high and low pFe values, which could lead to synergistic ligand promoted Fe dissolution (Fig. 2a). Interestingly, if driven by the early release, the control of this process would occur via the selection of biosynthetic enzymes that produce more or less catechol fragments rather than the direct regulation afforded

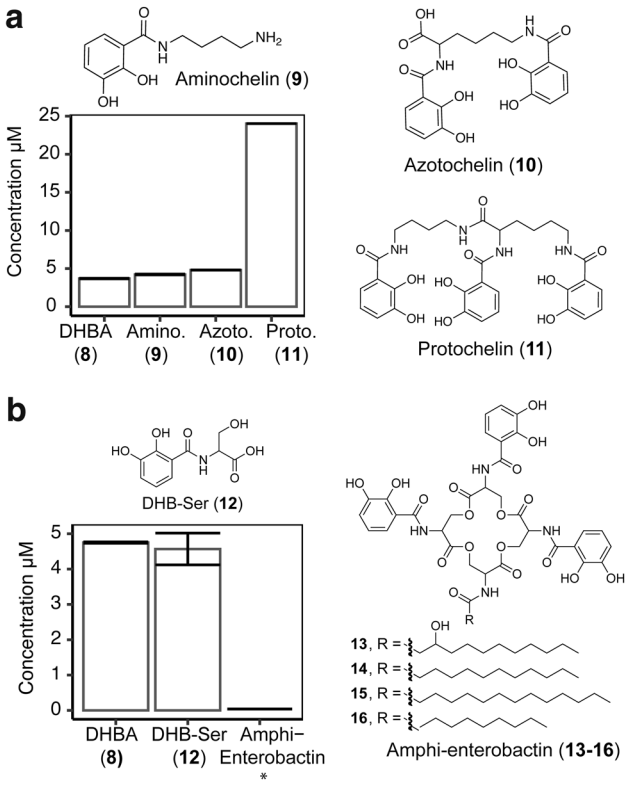

Fig. 4 **a** Tris-, bis-, and mono- catechol fragments released by *Azotobacter vinelandii* and concentrations. *A. vinelandii* cells were grown with 500 nM Fe and 100 µM EDTA for 37 h [49]. **b** Tris- and mono-catechol fragments released by *Vibrio harveyi* and concentrations. *V. harveyi* cells were grown with 100 nM Fe and 100 µM EDTA for 24 h [82]. To avoid complications from quorum sensing (QS) repression, a mutant (*luxO* D47E [122]) that does not respond to QS was used. *Data shown are for amphi-enterobactin variant $m/z = 965.3681$, for which a structure has not been confirmed

by encoding multiple gene clusters. Furthermore, the extent to which fragments are released may vary across organisms. Despite extensive in vitro studies of model catechol biosynthesis (such as that of enterobactin), the reasons for fragment release in vivo are not well known. Simple measurements of fragment concentrations across multiple organisms or growth stages are now easy to conduct and would provide useful information about the utility of these smaller siderophores as well as in vivo siderophore production.

Variations in siderophores based on intracellular precursor pools

The intracellular concentration of siderophore precursors is another factor that can influence the types of siderophore structures that are synthesized. While this mechanism is likely to lead to only small structural variations, many of these can still be biologically relevant. Precursor-directed

biosynthesis (PDB) has been appreciated by natural products chemists for some time [103]. We focus on three examples where MSP could be driven by fluctuations in intracellular precursors: the synthesis of amphiphilic siderophores with fatty acid tails of variable length, the incorporation of different polyamine precursors into hydroxamate siderophores, and the in vitro production of three mixed functionality siderophores by the same biosynthetic gene cluster.

Amphiphilic siderophores were first identified in pathogenic terrestrial bacteria [104]. However, subsequent research has shown extremely common production of amphiphilic siderophores in marine organisms, including aquachelins [105], marinobactins [105], amphibactins [50], ochrobactins [106], and synechobactins [107]. These siderophores are typically produced as suites of related compounds that share a head group but have hydrophobic tails of variable length, hydroxylation, and unsaturation. *Vibrio harveyi* amphi-enterobactins (13–16), discovered by Zane et al. in 2014 [95], are a recent addition to the suite of amphiphilic marine siderophores. Consistent with the previous findings, *V. harveyi* synthesizes amphi-enterobactins with variable tail lengths (Fig. 4b).

It has been proposed that the use of fatty acid tails provides an advantage in the marine environment by reducing losses to diffusion and that the production of siderophores with variable tail lengths might allow for a bucket brigade mechanism that shuttles Fe between more soluble siderophores (with shorter tails) to less soluble siderophores (with longer tails) at the cell surface [50]. The membrane partitioning of amphiphilic siderophores has been studied [50, 55, 106], and while the head group has an important role in membrane association, siderophores with longer tail lengths are generally found to have greater membrane partitioning. Amphiphilic siderophores have also been shown to form micelles at high concentrations, and iron acquisition via this mechanism has been proposed [105]. The production of suites of amphiphilic siderophores is clearly widespread. Key unanswered questions are the extent to which the distribution of tail lengths is regulated as well as the potential benefits conferred. However, changes in the intracellular fatty acid pool due to metabolic or environmental fluctuations (in temperature, for example [108]) could influence amphiphilic siderophore production.

Another example of precursor-directed biosynthesis is the incorporation of different polyamines in hydroxamate siderophores. Early studies of desferrioxamine biosynthesis in *Streptomyces olivaceus* were able to elicit the production of multiple related siderophores by changing the polyamine substrates [103, 109, 110]. Similarly, in vitro studies of a *Shewanella algae* hydroxamate siderophore gene cluster that incorporates putrescine (**17**) and cadaverine (**18**) were also able to produce putrebactin (**19**), avaroferrin (**20**), and bisucaberin (**21**) by varying the polyamine substrates [111]. Putrebactin utilizes two putrescine molecules, bisucaberin utilizes two cadaverine molecules, and the asymmetric avaroferrin incorporates one molecule of each (Fig. 5). These findings have been extended to in vivo studies. When grown in medium without added polyamines, *Shewanella algae* synthesized putrebactin, avaroferrin, and bisucaberin in ratios of 1:2:1. However, when the medium was supplemented with large excesses of either putrescine or cadaverine, biosynthesis was biased toward either putrebactin or bisucaberin, respectively [111, 112]. Despite their seemingly small structural variations, these siderophores showed biologically relevant differences. Avaroferrin halts swarming in *Vibrio alginolyticus,* whereas putrebactin and bisucaberin have a little effect on swarming motility. The reason for this difference appears to be Fe immobilization—all three siderophores have similar ferric stability constants, but slight structural differences render the avaroferrin–Fe complex inaccessible to *V. alginolyticus* [29]. Studies of PDB in *S. algae* have also demonstrated the incorporation of non-natural precursors [113], illustrating the flexibility of siderophore biosynthesis in this organism. This flexibility provides a direct advantage to *S. algae* allowing it to avoid siderophore theft or to starve *V. alginolyticus* via competitive Fe chelation.

A third example of precursor incorporation involves the mixed functionality siderophores prepseudomonine (**22**), pseudomonine (**23**), preacinetobactin (**24**), acinetobactin (**25**), and anguibactin (**26**) (Fig. 6). The pseudomonine biosynthetic assembly line has been purified from *Pseudomonas entomophila*. When salicylic acid, threonine, and histamine are used as building blocks, this assembly line produces prepseudomonine, which re-arranges non-enzymatically to form pseudomonine [114, 115]. However, the use of DHBA instead of salicylic acid results in the production of preacinetobactin, which also re-arranges non-enzymatically (and analogously) to form acinetobactin [115]. The use of cysteine as a substrate yields the production of anguibactin (a siderophore from *Vibrio anguillarum* [116]), which does not undergo non-enzymatic re-arrangement [115]. This

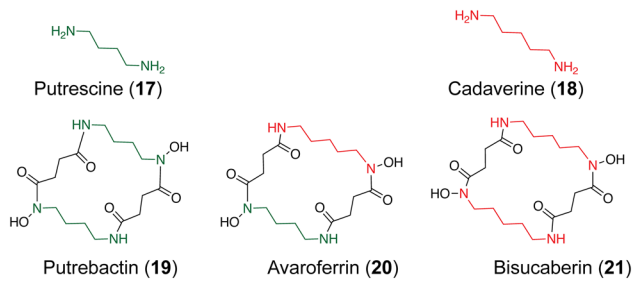

Fig. 5 Structures of putrebactin, avaroferrin, and bisucaberin, which are produced from different combinations of putrescine and cadaverine

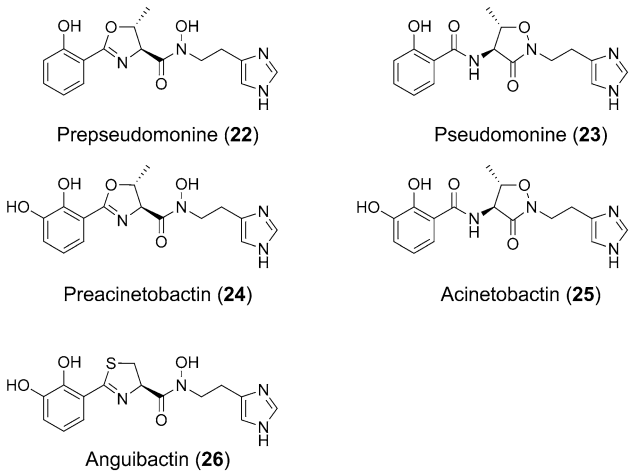

Fig. 6 Structures of prepseudomonine, pseudomonine, preacinetobactin, acinetobactin, and anguibactin. All siderophore arise from the sample gene cluster. Prepseudomonine and preacinetobactin rearrange non-enzymatically to form pseudomonine and acinetobactin, respectively [115]

finding has not been explored in vivo, although there has been some investigation of the functionality of preacinetobactin and acinetobactin. The pH-dependent transition from preacinetobactin to acinetobactin has been proposed to allow the pathogen *Acinetobacter baumannii* to obtain iron under varying pH conditions [117]. If these findings hold in vivo, changes in the intracellular amino acid concentrations could lead to the biosynthesis of these three different siderophores by a single organism using one gene cluster.

The metabolic status of the cell is likely to affect intracellular precursor pools and siderophore production in interesting ways. For example, polyamines help microbes to cope with oxidative stress and have hypothesized roles in pathogenicity [118]. Increases in total polyamine concentrations or variations in the distributions of polyamines could, therefore, affect the siderophore variants released under different conditions. In cases where siderophore biosynthesis is well-understood and small structural variants have already been documented, explorations of environmental controls on this variation are now feasible and may offer insight into the ways that interactions between the environment and microbial metabolism drive small-molecule production.

Conclusions

Since the initial discovery of siderophores in the 1950s [119–121], much has been learned about the biosynthesis and structures of these molecules. Multiple siderophore production is a recurrent theme in siderophore studies and has consequences for microbial interactions and trace metal acquisition. Multiple siderophores can help microbes to avoid siderophore theft or facilitate competition with other organisms. Co-production of siderophores with high and low Fe affinities may enhance Fe dissolution through ligand exchange, while hydrophilic and hydrophobic siderophores may participate in a bucket brigade to shuttle Fe from the bulk medium to the cell. In addition, there is growing evidence for non-classical functions for siderophores such as iron storage and use in the acquisition of other trace elements. Most simply put, studies suggest that multiple siderophores exist because they have a function: they work synergistically or contingently [4] to provide Fe to the cell, or have important roles in other non-Fe-related processes.

The mechanisms by which multiple siderophores are synthesized may also provide varied levels of control on siderophore production, a feature that could be useful in different environmental contexts. Encoding multiple biosynthetic gene clusters allows for transcriptional regulation of siderophores in response to the established environmental cues such as iron concentration. In contrast, fragment release by the catechol biosynthetic machinery could be selected over evolutionary time scales. Yet, a third level of control is provided by precursor-directed biosynthesis which ties siderophore production directly to environmental and metabolic conditions, avoiding both the increased specificity and restrictions of transcriptional regulation. On this basis, a few general advantages of hydroxamate vs. catechol siderophore biosynthesis can be proposed. Catechol production may offer a way to release suites of siderophores with both high and low Fe affinities as well as hydrophobic and hydrophilic siderophores. Hydroxamate siderophores may be particularly sensitive to the composition of intracellular precursor pools, allowing for more dynamic responses to changing conditions.

Unraveling the function of multiple siderophore production is one of the key future challenges and opportunities in siderophore research. Several mechanisms for MSP exist and general trends associated with each are beginning to emerge, with diversity as the main theme. Even in cases where organisms encode multiple gene clusters, it is clear that siderophores are typically produced in suites. For example, *P. aeruginosa,* which synthesizes pyoverdine and pyochelin, not only makes siderophores with variable pFe values, it also makes several versions of each. As discussed above, focusing on small structural variants is often necessary to understand biological relevance. It is also becoming clear that the existence of a pool of siderophores with slight differences is often beneficial for the producing organism. Accordingly, variations in siderophore diversity among organisms may provide revealing insights. Finally, as illustrated by the examples of precursor-directed biosynthesis, environmental conditions and biosynthetic machinery may be equally important in dictating which siderophores are

released. In light of these considerations, siderophore production appears to be a process driven not by the organism or the environment alone but rather by ever-changing combinations of the two.

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References

1. Williams DH, Stone MJ, Hauck PR, Rahman SK (1989) J Nat Prod 52:1189–1208
2. Firn RD, Jones CG (2003) Nat Prod Rep 20:382–391
3. Fischbach MA, Clardy J (2007) Nat Chem Biol 3:353–355
4. Challis GL, Hopwood DA (2003) Proc Natl Acad Sci USA 100:14555–14561
5. Hantke K (1981) Mol Gen Genet 182:288–292
6. Zimmermann L, Hantke K, Braun V (1984) J Bacteriol 159:271–277
7. Bagg A, Neilands JB (1987) Biochemistry 26:5471–5477
8. Crumbliss AL, Harrington JM (2009) Adv Inorg Chem 61:179–250
9. Sandy M, Butler A (2009) Chem Rev 109:4580–4595
10. Hider RC, Kong X (2010) Nat Prod Rep 27:637–657
11. Kraemer SM (2004) Aquat Sci 66:3–18
12. Crosa JH (1989) Microbiol Rev 53:517–530
13. Crosa JH, Walsh CT (2002) Microbiol Mol Biol Rev 66:223–249
14. Challis GL (2005) Chem Bio Chem 6:601–611
15. Fischbach MA, Walsh CT (2006) Chem Rev 106:3468–3496
16. Luckey M, Pollack JR, Wayne R, Ames BN, Neilands JB (1972) J Bacteriol 111:731–738
17. Granger J, Price NM (1999) Limnol Oceanogr 44:541–555
18. Loper JE, Henkels MD (1999) Appl Environ Microbiol 65:5357–5363
19. Yamanaka K, Oikawa H, Ogawa H-O, Hosono K, Shinmachi F, Takano H, Sakuda S, Beppu T, Ueda K (2005) Microbiology 151:2899–2905
20. D'Onofrio A, Crawford JM, Stewart EJ, Witt K, Gavrish E, Epstein S, Clardy J, Lewis K (2010) Chem Biol 17:254–264
21. Cordero OX, Ventouras L-A, DeLong EF, Polz MF (2012) Proc Natl Acad Sci USA 109:20059–20064
22. Miethke M, Kraushaar T, Marahiel MA (2013) FEBS Lett 587:206–213
23. Tanabe T, Funahashi T, Miyamoto K, Tsujibo H, Yamamoto S (2011) Biol Pharm Bull 34:570–574
24. Traxler MF, Seyedsayamdost MR, Clardy J, Kolter R (2012) Mol Microbiol 86:628–644
25. Galet J, Deveau A, Hôtel L, Frey-Klett P, Leblond P, Aigle B (2015) Appl Environ Microbiol 81:3132–3141
26. Bister B, Bischoff D, Nicholson GJ, Valdebenito M, Schneider K, Winkelmann G, Hantke K, Süssmuth RD (2004) Biometals 17:471–481
27. Fischbach MA, Lin H, Liu DR, Walsh CT (2005) Proc Natl Acad Sci 102:571–576
28. Fischbach MA, Lin H, Liu DR, Walsh CT (2006) Nat Chem Biol 2:132–138
29. Böttcher T, Clardy J (2014) Angew Chem Int Ed Engl 53:3510–3513
30. Ishida S, Arai M, Niikawa H, Kobayashi M (2011) Biol Pharm Bull 34:917–920
31. Deveau A, Gross H, Palin B, Mehnaz S, Schnepf M, Leblond P, Dorrestein PC, Aigle B (2016) FEMS Microbiol Ecol 92:fiw107
32. Amin SA, Green DH, Hart MC, Küpper FC, Sunda WG, Carrano CJ (2009) Proc Natl Acad Sci USA 106:17071–17076
33. Guan LL, Kanoh K, Kamino K (2001) Appl Environ Microbiol 67:1710–1717
34. Johnstone TC, Nolan EM (2015) Dalton Trans 44:6320–6339
35. Adler C, Corbalán NS, Seyedsayamdost MR, Pomares MF, de Cristóbal RE, Clardy J, Kolter R, Vincent PA (2012) PLoS One 7:e46754
36. Charlang GW, Horowitz NH (1971) Proc Natl Acad Sci 68:260–262
37. Charlang G, Ng B, Horowitz NH, Horowitz RM (1981) Mol Cell Biol 1:94–100
38. Haas H (2014) Natural Product Reports 31:1266–1276
39. Haas H (2003) Appl Microbiol Biotechnol 62:316–330
40. Johnson L (2008) Mycol Res 112:170–183
41. Kraepiel AML, Bellenger JP, Wichard T, Morel FMM (2009) Biometals 22:573–581
42. Kraemer SM, Duckworth OW, Harrington JM, Schenkeveld WDC (2015) Aquat Geochem 21:159–195
43. Springer SD, Butler A (2016) Coord Chem Rev 306:628–635
44. Kenney GE, Sadek M, Rosenzweig AC (2016) Metall Integr Biometal Sci 8:931–940
45. Balasubramanian R, Kenney GE, Rosenzweig AC (2011) J Biol Chem 286:37313–37319
46. Bellenger JP, Wichard T, Kraepiel AML (2008) Appl Environ Microbiol 74:1478–1484
47. Bellenger JP, Wichard T, Kustka AB, Kraepiel AML (2008) Nat Geosci 1:243–246
48. Wichard T, Bellenger JP, Loison A, Kraepiel AML (2008) Environ Sci Technol 42:2408–2413
49. McRose DL, Baars O, Morel FMM, Kraepiel AML (2017) Environ Microbiol 48:11451–13605
50. Martinez JS, Carter-Franklin JN, Mann EL, Martin JD, Haygood MG, Butler A (2003) Proc Natl Acad Sci USA 100:3754–3759
51. Homann VV, Edwards KJ, Webb EA, Butler A (2009) BioMetals 22:565–571
52. Gauglitz JM, Iinishi A, Ito Y, Butler A (2014) Biochemistry 53:2624–2631
53. Ratledge C, Ewing M (1996) Microbiology 142:2207–2212
54. Gobin J, Horwitz MA (1996) J Exp Med 183:1527–1532
55. Xu G, Martinez JS, Groves JT, Butler A (2002) J Am Chem Soc 124:13408–13415
56. Reichard P, Kretzschmar R, Kraemer S (2007) Geochim Cosmochim Acta 71:5635–5650
57. Cheah S-F, Kraemer SM, Cervini-Silva J, Sposito G (2003) Chem Geol 198:63–75
58. Cox CD, Adams P (1985) Infect Immun 48:130–138
59. Albrecht-Gary AM, Blanc S, Rochel N, Ocaktan A, Abdallah M (1994) Inorg Chem 33:6391–6402
60. Cox CD, Graham R (1979) J Bacteriol 137:357–364
61. Brandel J, Humbert N, Elhabiri M, Schalk IJ, Mislin GLA, Albrecht-Gary A-M (2012) Dalton Trans 41:2820–2834
62. Meyer JM, Van VT, Stintzi A, Berge O, Winkelmann G (1995) Biometals 8:309–317
63. Meyer JM, Hohnadel D, Hallé F (1989) J Gen Microbiol 135:1479–1487
64. Boukhalfa H, Crumbliss AL (2002) Biometals 15:325–339
65. Nurchi VM, Pivetta T, Lachowicz JI, Crisponi G (2009) J Inorg Biochem 103:227–236
66. Sokol PA, Lewis CJ, Dennis JJ (1992) J Med Microbiol 36:184–189
67. Bulen WA, LeComte JR (1962) Biochem Biophys Res Commun 9:523–528

68. Page WJ, Collinson SK, Demange P, Dell A, Abdallah MA (1991) Biol Metals 4:217–222
69. Baars O, Zhang X, Gibson MI, Stone AT, Morel FMM, Seyedsayamdost MR (2017) Angew Chem Int Ed Engl. https://doi.org/10.1002/anie.201709720
70. Baars O, Zhang X, Morel FMM, Seyedsayamdost MR (2015) Appl Environ Microbiol 82:27–39
71. Cornish AS, Page WJ (1998) Microbiology 144:1747–1754
72. Corbin JL, Bulen WA (1969) Biochemistry 8:757–762
73. Cornish AS, Page WJ (1995) Biometals 8:332–338
74. Hider RC, Liu ZD (2004) In: Atwood JL, Steed JW (eds) Encyclopedia of supramolecular chemistry. Taylor and Francis, Boca Raton, pp 1278–1290
75. Khodr H, Hider R, Duhme-Klair AK (2002) J Biol Inorg Chem 7:891–896
76. Teitzel GM, Geddie A, De Long SK, Kirisits MJ, Whiteley M, Parsek MR (2006) J Bacteriol 188:7242–7256
77. Martin LW, Reid DW, Sharples KJ, Lamont IL (2011) Biometals 24:1059–1067
78. Izrael-Živković L, Rikalović M, Gojgić-Cvijović G, Kazazić S, Vrvić M, Brčeski I, Beškoski V, Lončarević B, Gopčević K, Karadžić I (2018) RSC Advances 8:10549–10560
79. Dumas Z, Ross-Gillespie A, Kümmerli R (2013) Proc R Soc Lond B Biol Sci 280:20131055
80. Lamont IL, Beare PA, Ochsner U, Vasil AI, Vasil ML (2002) Proc Natl Acad Sci 99:7072–7077
81. Dietrich LEP, Price-Whelan A, Petersen A, Whiteley M, Newman DK (2006) Mol Microbiol 61:1308–1321
82. McRose D, Baars O, Seyedsayamdost MR, Morel FMM (2018) Proc Natl Acad Sci 115:7581–7586
83. Persmark M, Neilands JB (1992) Biometals 5:29–36
84. Sandy M, Butler A (2011) J Nat Prod 74:1207–1212
85. Sandy M, Han A, Blunt J, Munro M, Haygood M, Butler A (2010) J Nat Prod 73:1038–1043
86. Han AW, Sandy M, Fishman B, Trindade-Silva AE, Soares CAG, Distel DL, Butler A, Haygood MG (2013) PLoS One 8:e76151
87. O'Brien IG, Gibson F (1970) Biochimica Et Biophysica Acta 215:393–402
88. Harris WR, Carrano CJ, Cooper SR, Sofen SR, Avdeef AE, McArdle JV, Raymond KN (1979) J Am Chem Soc 101:6097–6104
89. Bryce GF, Brot N (1972) Biochemistry 11:1708–1715
90. Langman L, Young IG, Frost GE, Rosenberg H, Gibson F (1972) J Bacteriol 112:1142–1149
91. Greenwood KT, Luke RK (1978) Biochem Biophys Acta 525:209–218
92. Brickman TJ, McIntosh MA (1992) J Biol Chem 267:12350–12355
93. Lin H, Fischbach MA, Walsh CT (2005) J Am Chem Soc 127:11075–11084
94. Reitz ZL, Sandy M, Butler A (2017) Metallomics 9:824–839
95. Zane HK, Naka H, Rosconi F, Sandy M, Haygood MG, Butler A (2014) J Am Chem Soc 136:5615–5618
96. Beld J, Sonnenschein EC, Vickery CR, Noel JP, Burkart MD (2014) Nat Prod Rep 31:61–108
97. Ratledge C, Winder FG (1962) Biochem J 84:501–506
98. Ratledge C, Hall MJ (1971) J Bacteriol 108:314–319
99. Lebeis SL, Paredes SH, Lundberg DS, Breakfield N, Gehring J, McDonald M, Malfatti S, del Rio TG, Jones CD, Tringe SG, Dangl JL (2015) Science 349:8764–8864
100. Bellenger JP, Wichard T, Xu Y, Kraepiel AML (2011) Environ Microbiol 13:1395–1411
101. Hancock RE, Hantke K, Braun V (1977) Arch Microbiol 114:231–239
102. Hantke K (1990) FEMS Microbiol Lett 67:5–8
103. Thiericke R, Rohr J (1993) Nat Prod Rep 10:265–289
104. Francis J, Macturk HM, Madinaveitia J, Snow GA (1953) Biochem J 55:596–607
105. Martinez JS, Zhang GP, Holt PD, Jung HT, Carrano CJ, Haygood MG, Butler A (2000) Science 287:1245–1247
106. Martin JD, Ito Y, Homann VV, Haygood MG, Butler A (2006) J Biol Inorg Chem 11:633–641
107. Ito Y, Butler A (2005) Limnol Oceanogr 50:1918–1923
108. Neidleman S (1987) Biotechnol Genet Eng Rev 5:245–268
109. Konetschny-Rapp S, Jung G, Raymond K, Meiwes J, Zähner H (1992) J Am Chem Soc 114:2224–2230
110. Schafft M, Diekmann H (1978) Arch Microbiol 117:203–207
111. Rütschlin S, Gunesch S, Böttcher T (2017) Cell Chem Biol. https://doi.org/10.1016/j.chembiol.2017.03.017
112. Soe CZ, Telfer TJ, Levina A, Lay PA, Codd R (2016) J Inorg Biochem 162:207–215
113. Rütschlin S, Gunesch S, Böttcher T (2018) ACS Chem Biol 13:1153–1158
114. Sattely ES, Walsh CT (2008) J Am Chem Soc 130:12282–12284
115. Wuest WM, Sattely ES, Walsh CT (2009) J Am Chem Soc 131:5056–5057
116. Actis LA, Fish W, Crosa JH, Kellerman K, Ellenberger SR, Hauser FM, Sanders-Loehr J (1986) J Bacteriol 167:57–65
117. Shapiro JA, Wencewicz TA (2015) ACS Infect Dis 2:157–168
118. Shah P, Swiatlo E (2008) Mol Microbiol 68:4–16
119. Francis J, Madinaveitia J, Macturk HM, Snow GA (1949) Nature 163:365–366
120. Neilands JB (1952) J Am Chem Soc 74:4846–4847
121. Hesseltine CW, Pidacks C, Whitehill AR, Bohonos N, Hutchings B, WIlliams JH (1952) J Am Chem Soc 74:1362–1363
122. Lilley BN, Bassler BL (2000) Mol Microbiol 36:940–954