Mining elements of siderophore chirality encoded in microbial genomes

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Abbreviations:
antiSMASH, antibiotics & Secondary Metabolite Analysis Shell
BGC, biosynthetic gene cluster
CAA, cationic amino acid
DHB, dihydroxybenzoate
NRPS, nonribosomal peptide synthetase
Orn, ornithine

Abstract

The vast majority of bacteria require iron to grow. A significant iron acquisition strategy is the production of siderophores, which are secondary microbial metabolites synthesized to sequester iron(III). Siderophore structures encompass a variety of forms, of which highly modified peptidic siderophores are of interest herein. State-of-the-art genome mining tools, such as antiSMASH (antibiotics & Secondary Metabolite Analysis SHell) hold the potential to predict and discover new peptidic siderophores, including a combinatoric suite of triscatechol siderophores framed on a triserine-ester backbone of the general class, (DHB-$^{L/D}$CAA-^{L}Ser)$_3$ (CAA, cationic amino acid). Siderophores with $^{L/D}$Arg, $^{L/D}$Lys and ^{L}Orn, but not ^{D}Orn, were predicted in bacterial genomes. Fortuitously the ^{D}Orn siderophore was identified, yet its lack of prediction highlights the limitation of current genome mining tools. The full combinatoric suite of these siderophores, which form chiral iron(III) complexes, reveals stereospecific coordination chemistry encoded in microbial genomes. The chirality embedded in this suite of Fe(III)-siderophores raises the question of whether the relevant siderophore-mediated iron acquisition pathways are stereospecific and selective for ferric siderophore complexes of a defined configuration.

Iron is essential for the growth of most bacteria, with the possible exception of a small number of species.[1, 2] Given the importance of iron, bacteria have evolved multiple pathways to acquire iron depending on the environment in which they are living. Some bacteria obtain iron from Fe-Heme or the iron transport proteins transferrin or lactoferrin, and some bacteria may utilize cell-surface ferric-reductases to produce Fe(II) for subsequent uptake. Yet a particularly significant pathway for iron uptake is via microbial production of siderophores – natural products that coordinate Fe(III) with exceptional affinity and which are recognized by specific bacterial outer membrane receptor proteins for cellular internalization.

Hundreds of siderophore structures are known.[3] The vast majority of siderophores were discovered by direct isolation from microbial cultures. However, the exponential increase in sequencing capabilities has revolutionized the discovery of natural products in tandem with dramatic advancements in the bioinformatic tools used to mine microbial genomes. The antiSMASH (antibiotics & Secondary Metabolite Analysis SHell) platform is essential for the analysis of secondary metabolites and is a powerful predictive tool for identifying potentially novel biosynthetic gene clusters (BGCs).[4] AntiSMASH is particularly useful in identifying and analyzing nonribosomal peptide synthetase (NRPS) enzymes that synthesize microbial peptide natural products, including peptidic siderophores. Additionally, antiSMASH and related bioinformatic tools can be leveraged to make *de novo* structural predictions for the secondary metabolites associated with their corresponding BGC.

Although a universal siderophore biosynthetic gene cluster does not exist, biosynthetic gene clusters encoding enzymes that synthesize siderophores are readily identified by screening for the genes involved in the biosynthesis of known Fe(III)-coordinating functional groups. These ligands include catechols, hydroxamic acids, α-hydroxycarboxylic acids, thiazoles, oxazoles and, the recently discovered, diazeniumdiols. Much is known about the biosynthetic routes of these ligands, including the catechol 2,3-dihydroxybenzoic acid (DHBA),[5] acetylated or formylated N-OH-ornithine and N-OH-lysine residues,[6 7] the α-hydroxy acids β-OH-aspartic acid and β-OH-histidine,[8] and the thiazole and oxazole groups,[9] all of which are components of peptidic siderophores. At this point, little is known about the biosynthesis of the N-N-bonded diazeniumdiolate group in the amino acid graminine found in certain peptidic siderophores.[10 11]

The biosynthetic gene cluster for the triscatechol siderophore enterobactin is well established in *E. coli* and consists of the NRPS, EntF, with a C-start domain and an adenylation domain specific for ^{L}Ser (Figure 1 and see the Boxed Highlight describing the NRPS architecture and functional domains within NRPS).[5] It operates by loading ^{L}Ser and appending DHB to the amine of ^{L}Ser in an iterative fashion to produce the macrolactone trimer (DHB-^{L}Ser)$_3$, i.e., enterobactin. The NRPS for amphi-enterobactin, AebF, found in several *Vibrio* species is related to EntF, but is distinguished by producing a tetraserine macrolactone in which one Ser-amine is appended by a fatty acid (Figure 1).[12] AebF (*V. campbellii* BAA1116) is 36% identical to EntF (*E. coli* UTI89) and was originally annotated as EntF, however it does not produce enterobactin.[12 13] Instead biosynthesis of amphi-enterobactin is initiated by the fatty acyl CoA ligase, AebG ligating a fatty acid to the first loaded ^{L}Ser; then subsequently, AebF adds three DHB-^{L}Ser

groups in an iterative fashion to yield a unique tetra ^{L}Ser macrolactone siderophore appended by one FA and three DHB catechols. No clues are evident to suggest that AebF would not synthesize enterobactin, although the lack of production of enterobactin and the presence of a fatty acid CoA ligase near the BGC of *V. campbellii* BAA1116 provided the impetus to look for a fatty acyl derivative of enterobactin.[12]

The related triscatechol siderophore bacillibactin (BB; Figure 1) – the macrolactone of (DHB-Gly-^{L}Thr)$_3$ – contains an expanded NRPS, DhbF,[14] incorporating a second adenylation domain responsible for loading Gly and inserting it between the Thr macrolactone and the catechol binding groups. Similar to the two-module NRPS of bacillibactin, the siderophores trivanchrobactin, cyclic trichrysobactin and turnerbactin, all of which were discovered through untargeted analysis of microbial extracts, require expanded, two-module NRPS enzymes to incorporate ^{D}Arg (trivanchrobactin), ^{D}Lys (cyclic trichrysobactin) or ^{L}Orn (turnerbactin) between DHB and ^{L}Ser (Figure 2). Chiral siderophores are notable in that they frequently coordinate Fe(III) in a stereospecific manner with a defined preference for either the Δ or Λ configuration. For example, FeEnt^{3-} with ^{L}Ser and the synthetic FeEnantioEnt^{3-} with ^{D}Ser adopt Δ or Λ configurations, respectively,[15] while bacillibactin, with Gly and ^{L}Ser, adopts the Λ configuration on Fe(III) coordination.[16] The presence of cationic amino acids with variable stereochemistry immediately adjacent to the Fe(III) coordinating catechol groups raises questions concerning the ability of chirality embedded within the ligand to direct the resulting configurational preference of the corresponding iron complexes.

To date, over a million bacterial genomes have been sequenced. NCBI RefSeq has established a list of *ca* 15,000 high quality genomes to represent known bacterial diversity.[17] These genomes can first be screened for homologs of the *entABCE* set of genes used in the biosynthesis of 2,3-DHB (Figure 2a), and the resulting subset screened for those containing two-module NRPSs consistent with triscatechol siderophores, in which the 1st module begins with a C_{Start} domain used to append DHB. Further rounds of screening can be conducted to identify NRPS enzymes containing an adenylation domain with Stachelhaus codes[18] specific for ^{L}Lys, ^{L}Arg or ^{L}Orn, and a 2nd module that contains an adenylation domain specific for ^{L}Ser (Figure 2a,b). If an epimerization (E) domain is present in the 1st domain, the L-amino acid loaded is converted to a D-amino acid prior to condensation. Thus, for trivanchrobactin,[19] cyclic trichrysobactin,[20] and turnerbactin,[21] analysis of the NRPS following structural characterization of the natural products, revealed a domain architecture consistent with the identity and stereochemistry of the amino acids incorporated into the siderophores (Fig 2b, c).

The genome mining workflow described previously was useful not only in verifying these characterized natural products, but more importantly in identifying putative homologs, particularly stereochemical isomers, that had yet to be identified in nature. We set out to search for the full combinatoric suite of $^{D/L}$Lys, $^{D/L}$Arg and $^{D/L}$Orn siderophores with an ultimate goal to investigate these structural predictions and uncover the consequences of chirality in siderophore-mediated iron acquisition (Figure 1b).

We have now isolated and characterized the predicted siderophores with ^{L}Arg from the fish pathogen *Yersinia ruckeri* YRB,[22] and ^{L}Lys from the human opportunistic pathogen *Yersinia*

frederiksenii ATCC 33641.[23] *Y. ruckeri* YRB produces the triscatechol siderophore ruckerbactin which is the linear ^{L}Ser oligoester, (DHB-^{L}Arg-^{L}Ser)$_3$ (Figure 2c). The NRPS RucF in the BGC of *Y. ruckeri* YRB lacks an epimerization domain for ^{L}Arg; thus, ruckerbactin with ^{L}Arg is the diastereomer of trivanchrobactin produced by *V. campbellii* DS40M4 with ^{D}Arg.[22 19] *Y. frederiksenii* ATCC 33641 produces frederiksenibactin, the linear ^{L}Ser oligoester, (DHB-^{L}Lys-^{L}Ser)$_3$ (Figure 1c). Similarly, the NRPS enzyme FreF in the BGC of *Y. frederiksenii* ATCC 33641 lacks an epimerization domain retaining the chirality of the loaded ^{L}Lys. In contrast, an epimerization domain is present in the NRPS CbsF of *Dickeya chrysanthemi* EC16 responsible for biosynthesis of the ^{D}Lys homolog cyclic trichrysobactin and hence the two siderophores incorporate Lys into their structures with opposing stereochemistry. Frederiksenibactin and CTC are not true diastereomers, with one residing as a linear oligoester and one as a macrolactone (Figure 2c).[23 20] Presently, the factors guiding macrolactonization at the level of the thioesterase domain are not understood, and bioinformatic tools are limited in their ability to predict whether the NRPS-derived natural product is released as a linear carboxylic acid or a cyclic lactone.

The discovery of diastereomeric pairs of siderophores harboring L- and D- Arg or L- and D-Lys provided a tantalizing hint that the stereochemistry of Fe(III) coordination may be directed by the stereochemistry of the cationic amino acid. In fact the electronic circular dichroism (ECD) spectra do reveal that the chirality of the amino acid appended to the DHB catechol sets the configuration at the Fe(III) site.[23] Fe(III)-trivanchrobactin with ^{D}Arg and Fe(III)-cyclic-

trichrysobactin with ^{D}Lys form the Λ complex, while Fe(III)-ruckerbactin with ^{L}Arg and Fe(III)-frederiksenibactin with ^{L}Lys form Δ complexes (Figure 2c).

Turnerbactin isolated from the shipworm symbiont, *Teredinibacter turnerae* T7901 is the ^{L}Orn-containing triscatechol siderophore framed on the linear ^{L}Ser oligoester, (DHB-^{L}Orn-^{L}Ser)$_3$ (Figure 3a).[21] To fill out the combinatoric suite, we were interested in identifying bacterial producers of the hypothetical ^{D}Orn diastereomer; however, no reference genomes unambiguously predicted the ^{D}Orn siderophore. Fortuitously, we were investigating the siderophores from *Marinomonas mediterrranea* MMB-1. The genome encodes a two-module NRPS consistent with a triscatechol siderophore (Figure 3a). While module 1 contains an epimerization domain, the Stachelhaus code of the adenylation domain was a close match for Lys. Thus, *M. mediterranea* MMB-1 was predicted to produce a ^{D}Lys siderophore in the chrysobactin family. Instead, we found that the bacterium produces a small amount of a siderophore, mediterraneabactin, with the same MW as turnerbactin (m/z 1030.40 $[M+H]^+$; Figure 3b,d).

The presence of ^{D}Orn was established through derivatization with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, FDAA; Figure 3c).[25] Moreover, the ECD spectrum of Fe(III)-mediterraneabactin with ^{D}Orn reveals Λ complexation at Fe(III) (Figure 3e), which is consistent with other D-amino acid-containing triscatecholates. To further investigate the chirality of coordination with L- and D- Orn, the cyclic macrolactone (DHB-$^{L/D}$Orn-^{L}Ser)$_3$ diastereomers were synthesized using a synthetic scheme applied previously to access diastereomers of cyclic

trichrysobactin.[23] The cyclic analogs with ^{L}Orn and ^{D}Orn coordinate Fe(III) with Δ and Λ stereospecificity, respectively, and their ECD spectra are consistent with those of their linear analogs turnerbactin and mediterraneabactin (Figure 3f).

In sum, the identification of the combinatoric suite of triscatecholate siderophores (DHB-$^{L/D}$CAA-^{L}Ser)$_3$ (CAA, cationic amino acid) reveals stereospecific coordination chemistry encoded in microbial genomes. While bacterial culture isolation led to the discovery of trivanchrobactin, cyclic trichrysobactin and turnerbactin, genome mining was key to the discovery of ruckerbactin and frederiksenibactin. In both of these siderophores, the chirality and identity of the cationic spacer amino acid matched the genomic prediction. However, the ^{D}Orn spacer of mediterraneabactin was not accurately predicted by the Stachelhaus code. Identifying the adenylation domain selectivity remains an outstanding challenge in NRPS genome mining, and we have yet to find a tool that accurately predicts amino acid loading, as became evident in the BGC of *M. mediterranea* MMB-1. Thus, with the current state of bioinformatics tools, genome mining does not replace the requirement for experimental isolation and characterization of siderophores and other natural products. In fact, traditional methods of natural product characterization and verification of structural features are important in improving our predictive models. We have seen dramatic advancements in our ability to predict the structure of NRPS-derived natural products and further refinement of these models is in progress through correlation of structural data with genomic information.

Through use of the genome mining workflow described previously, we were able to isolate novel stereoisomers of known triscatecholate siderophores. Interestingly, these compounds are present as diastereomeric pairs of siderophores which are notably produced by different bacterial species. This observation raises the question of whether the relevant siderophore-mediated iron acquisition pathways are stereospecific and selective for ferric siderophore complexes of a defined chirality. Furthermore, these compounds coordinate Fe(III) in a stereoselective manner and their configurational preference is derived from the ligand stereochemistry encoded in the requisite biosynthetic machinery. Broader questions arise concerning the biological impact of metal-based chirality, as well as the evolutionary pressure driving the immense structural diversity observed in microbial metallophores.[26] The observed structural variation, particularly with regards to stereochemistry, may be a mechanism to privatize iron and produce sequestering ligands which are less exploitable by competing organisms.

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Figure Legends

Figure 1. Structures of enterobactin, amphi-enterobactin and bacillibactin showing a one-module NRPS architecture for EntF and ArbF and a two-module NRPS architecture for DhbF. See Boxed Highlight covering NRPS architectures.

Figure 2. Genome encoded combinatoric suite of triscatechol (DHB-$^{L-/D-}$CAA-^{L}Ser)$_3$ siderophores. (a) Biosynthesis of 2,3-DHB. (b) General structure depiction of the D-/L- CAA -containing siderophores and the NRPS domain architecture where the adenylation domain (lavender box) represents the selected amino acid (See Boxed Highlight for the NRPS functional domains). (c) Structures of the related $^{D-/L-}$Arg and $^{D-/L-}$Lys siderophores. The ECD spectra of the Fe(III)-complexes shows the ^{D}CAA directs formation the Λ Fe(III) conformer and the ^{L}CAA directs formation a Δ Fe(III) complex.

Figure 3. Prediction and characterization of turnerbactin diastereomer, mediterraneabactin. (a) NRPS domain architecture catalyzing biosynthesis of the triscatechol siderophores of mediterraneabactin and turnerbactin. The adenylation domains are represented by their amino

acid selectivity (i.e., Orn or Ser). (b) Structure of Mediterraneabactin. *Marinomonas mediterranea* MMB-1 was cultured in artificial seawater medium and mediterraneabactin was isolated as previously described for other siderophores.[24] (c) HPLC chromatogram of Marfey's assay for HCl hydrolysis product of mediterraneabactin co-injected with FDAA-^{D}Orn. (d) HR-ESI-MS spectrum of mediterraneabactin, *m/z* 1030.4014 $[M+H]^{1+}$. Calculated exact mass for mediterraneabactin $[M+H]^{1+}$ is *m/z* 1030.4006 ($C_{45}H_{60}N_9O_{19}$). (e) ECD spectra of Fe(III)-mediterraneabactin and Fe(III)-turnerbactin. (f) ECD spectra of synthesized Fe(III)-^{cyclic}(DHB-^{D}Orn-^{L}Ser)$_3$ and Fe(III)-^{cyclic}(DHB-^{L}Orn-^{L}Ser)$_3$.

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NRPS Architecture and Function of NRPS Domains.

NRPSs are modular enzymes comprised of multiple functional domains. The adenylation, **A**, domain (lavender box) recognizes and activates a specific amino acid (or other carboxylic acid) for transfer to the thiolation, **T**, domain (green box) as a thioester tethered via 4'-phosphopantetheine (Ppant). The condensation, **C**, domain (yellow box) catalyzes amide bond formation between two T-tethered units such as two amino acids or in the case of enterobactin, DHB-tethered to the T domain of the aryl carrier protein EntB and ^{L}Ser tethered to EntF.[5] An epimerization, **E**, domain (red box) adjacent to an A domain would convert the loaded L amino acid to the D-amino acid. The thioesterase, **Te**, domain (green box) is present in the terminal module of a NRPS and catalyzes hydrolysis of cyclization to release the final product. Amino acid selectivity in the **A** domain can be predicted by binding pocket residues, including a 10 amino acid sequence often called the Stachelhaus code.[18]