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Nonribosomal Peptides for Iron Acquisition: pyochelin biosynthesis as a case study

Trey A. Ronnebaum¹ and Audrey L. Lamb^{1,2}

¹Department of Chemistry, University of Kansas, Lawrence, Kansas 66045, United States

²Department of Molecular Biosciences, University of Kansas, Lawrence, Kansas 66045, United States

Abstract

Microbes synthesize small, iron-chelating molecules known as siderophores to acquire iron from the environment. One way siderophores are generated is by nonribosomal peptide synthetases (NRPSs). The bioactive peptides generated by NRPS enzymes have unique chemical features, which are incorporated by accessory and tailoring domains or proteins. The first part of this review summarizes recent progress in NRPS structural biology. The second part uses the biosynthesis of pyochelin, a siderophore from *Pseudomonas aeruginosa*, as a case study to examine enzymatic methods for generating the observed diversity in NRPS-derived natural products.

Graphical abstract

Keywords

siderophore; pyochelin; nonribosomal peptide synthetase; isochorismate synthase; isochorismate-pyruvate lyase; thiazoliny-imine reductase; stuffed domains; interrupted adenylation domains; methyltransferase; epimerase; yersiniabactin; watasemycin

Iron uptake: Siderophores

Iron is an essential nutrient that is needed by microbes to perform critical biological processes necessary for survival [1]. Due to the paucity of free iron in aerobic environments,

Corresponding Author: Audrey L. Lamb, lamb@ku.edu, phone: (785) 864-5075.

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microbes have developed intricate systems to acquire iron from their surroundings [1]. One such system is the biosynthesis of small molecules known as siderophores that have a high affinity for ferric iron [2]. When iron availability is low, microbes synthesize and secrete siderophores, and selectively reimport the iron-loaded form from the surrounding environment. Siderophore biosynthesis in bacteria is accomplished by nonribosomal peptide synthetase (NRPS) enzymes, polyketide synthase (PKS) enzymes, and/or by NRPS independent siderophore (NIS) synthetase enzymes [3–5]. Typically these enzymes do not have human homologues and knockdown experiments of siderophore biosynthesis have shown partial or complete suppression of pathogenicity for many different bacteria, making these enzymes attractive drug targets [6]. This review will provide an overview of the structural biology of NRPS enzymes and then focus on the structural and catalytic aspects of the enzymes involved in the biosynthesis of pyochelin, a siderophore from *Pseudomonas aeruginosa*, highlighting the involved accessory and tailoring activities that generate the unique chemical structure of pyochelin.

Nonribosomal peptide synthetases (NRPS): chemical logic of peptide chain formation

NRPS enzymes are large multidomain and multifunctional enzymes that display a chemical logic in which each module is responsible for the addition of a single amino acid to a growing peptide chain, including non-proteinogenic amino acids and hydroxy acids. An NRPS module consists of a condensation (C) domain, an adenylation (A) domain, and a peptidyl carrier protein (P) domain, except for the first initiation module, which lacks a condensation domain. The P-domain acts as a tethering system for the growing peptide chain, and must be post-translationally modified with coenzyme A on a conserved serine residue thereby generating a 4'phosphopantetheinyl (Ppant) swinging arm. A-domains have been named the “gate keepers” of the NRPS assembly line as they selectively activate and incorporate the appropriate amino acids into the growing peptide chain [7]. The A-domains activate an amino acid by catalyzing the formation of an aminoacyl-AMP, using ATP and releasing inorganic phosphate (Figure 1A). The Ppant thiol of the P-domain performs a nucleophilic attack on the carboxyl group of the aminoacyl-AMP, eliminating AMP and generating an aminoacyl-thioester bond, thereby priming the module. Once primed, the thioester tether on the P-domain transfers its amino acid cargo to subsequent domains, similar to a mechanical crane.

Peptide bond formation, or elongation, is catalyzed between the amino acids of two primed modules within the C-domain of the downstream module. The upstream thioester bond is broken as the peptide bond is formed, thereby transferring the elongating peptide to the downstream module. This process is repeated with each subsequent module until the full chain is made. Chain elongation is terminated by a thioesterase (T) domain in the terminal module [8]. The peptide is transferred from the thioester of the P-domain to a conserved serine residue of the T-domain, generating an amino ester, and allowing for hydrolysis and release of the mature peptide [8].

Nonribosomal peptide synthetases (NRPS): structural biology of an assembly line

The C-, A-, and P-domains have been well-studied, with both structural and kinetic characterizations [9–13]. The first structure elucidating the organization of a full NRPS module was the termination module (C-A-P-T) of surfactin biosynthesis, SrfA-C, from *Bacillus subtilis* (Figure 1B) [14]. Crystals of the full module were obtained after mutating the serine of the P-domain that is post-translationally modified with Ppant to alanine. The A-domain contains two subdomains, a larger N-terminal A core (A_{core}) domain and a smaller C-terminal A sub (A_{sub}) domain. The structure revealed a large interface between the C-domain and A_{core} -domain creating a platform for the A_{sub} - and P-domains. In this structure, the P-domain was oriented so that the tether would be directed towards the acceptor site of the C-domain.

Structures harboring the Ppant tether were documented with *holo*-AB3403 (C-A-P-T), a NRPS termination module from an uncharacterized biosynthetic pathway from *Acinetobacter baumannii* (AB3403, Figure 1C) and EntF from *E. coli* for the production of enterobactin (Figure 1D) [15**]. In AB3403, the C-domain adopted a closed conformation and the P-domain was found to be rotated $\sim 30^\circ$ in comparison to the *apo*-SrfA-C structure thereby promoting insertion of the Ppant into the C-domain. In EntF, the Ppant tether was directed into the adenylation domain, causing a shift in domain orientation of both the A_{sub} - and P-domains. Therefore, during the two different catalytic steps (adenylation and thiolation), the A_{sub} -domain assumes different orientations relative to the A_{core} -domain to promote the appropriate chemistries (Figure 1C–D) [16**]. This has been named “domain alternation” [17]. Importantly, when the Ppant tether is directed into the condensation domain for peptide bond formation, the A_{sub} -domain is in an adenylate-forming conformation, supporting hypotheses that while the peptide bond is being formed in the condensation domain for one biosynthetic product molecule, the A_{core} -domain adenylates an amino acid for the next catalytic cycle, suggesting NRPS enzymes work in true assembly line fashion [15**].

Domain alternation of A_{sub} - and P-domains were captured in multiple structures of LgrA, an NRPS of gramicidin synthase, representing the different steps of the catalytic cycle [16**]. LgrA is an initiation module containing a formyltransferase tailoring domain, an A-, and a P-domain. Structures of A_{sub} in the open and closed adenylation conformations were determined (Figure 1E) in which the P-domains were disordered. An isosteric analog mimicked the natural Ppant tether, replacing the hydrolyzable thioester with an amide (Val-NH-Ppant). With this analog, structures in the thiolation and formyltransferase conformations were determined in which the P-domain is ordered [16**,18]. In the thiolation state, the A_{sub} -domain was oriented so that the P-domain inserts the Ppant mimic into the adenylation domain extending evidence that A_{sub} -domain positioning is altered by the P-domain conformation. The differing thiolation and formyltransferase conformations of LgrA demonstrate the remarkable variability in module architecture [16**].

A cross-module structure consisting of an A- and P-domain from the first module of Dhbf and a C-domain from the second module was determined (Figure 1F) [19*]. Dhbf is a

dimodular NRPS involved in the biosynthesis of the *Bacillus* siderophore, bacillibactin. Unlike the observed interactions between A-domains and upstream C-domains, the downstream C-domain had no interaction with the upstream A-domain. In this structure, the C-domain only interacts with the “back” face of the P-domain as the Ppant tether was trapped in the thiolation state of the upstream adenylation domain by a mechanistic inhibitor. These data in addition to cryo-EM structures and modeling suggest a lack of conserved intermodular surface interactions [19*]. Additional higher-order structures examining intermodular interfaces may reveal specific structural interactions necessary for chain elongation.

MbtH-like proteins (MLPs) are auxiliary proteins sometimes found in NRPS gene clusters that have been hypothesized to have chaperone properties or to augment the catalytic activity of adenylation domains [20,21]. Crystal structures have been determined of MLPs alone and bound to A_{core}-domains [22–24*]. The DhbF structure described above was determined with an MLP bound to the A_{core}-domain (Figure 1F), and the MLP was shown to be essential for substrate binding and adenylation activity [19]. The full-length EntF NRPS module was co-crystallized with MLPs that enhance its adenylation activity: the MLP encoded within its operon, YbdZ (Figure 1D), and an MLP from *P. aeruginosa*, PA2412 [24*,25]. These structures exhibit no significant change in secondary structure of the A-domain upon MLP binding suggesting MLP binding may alter protein dynamics, though the mechanism by which MLPs enhance solubility or catalytic activity of A-domains remain elusive [19,24*].

Pyochelin from *Pseudomonas aeruginosa*

The chemical logic and structural biology of NRPS modules is key for understanding how the peptide bonds are made by these nanomachines. However, the bioactive peptides produced by these enzymes are far more diverse than the 20 amino acids found in proteins. The diversity is incorporated by accessory enzymes that produce unusual amino and hydroxy acid substrates for adenylation enzymes or by tailoring enzymes that alter amino acids already incorporated into the growing chain. Here, we use the biosynthesis of pyochelin, a small salicylate-capped, siderophore produced by *P. aeruginosa*, as an example to describe some of the accessory enzymes and tailoring domains involved in the biosynthesis of nonribosomal peptides.

Pyochelin is derived from three precursor molecules: one molecule of salicylate, a hydroxy acid, and two molecules of cysteine. Therefore, biosynthesis requires three NRPS modules: an initiation module that lacks a C-domain, an elongation module, and a termination module with a T-domain (Figure 2A). Biosynthesis of pyochelin is initiated by two accessory enzymes [26–28]. Chorismate is converted to isochorismate by the isochorismate synthase (ICS), PchA, [29] and the isochorismate is subsequently converted to salicylate by the isochorismate pyruvate lyase (IPL), PchB (Figure 2B) [30]. The salicylate is activated by the stand-alone A-domain, PchD, and transferred to the N-terminal P-domain of the NRPS, PchE (the initiation module) [31]. The elongation module of PchE integrates an L-cysteine into the growing peptide chain, cyclizes the L-cysteine to a thiazoline ring, and alters the stereochemistry with an epimerase tailoring domain [32]. The termination module of PchF adds and cyclizes a second L-cysteine to generate a second thiazoline. The tailoring protein

PchG reduces the thiazoline to thiazolidine [27]. The stuffed methyltransferase domain of PchF performs an *S*-adenosylmethionine (SAM) dependent *N*-methylation of the thiazolidine [33]. The mature pyochelin is released by the T-domain of PchF.

Accessory enzymes in pyochelin biosynthesis: PchA and PchB

PchA, isochorismate synthase

PchA catalyzes the first step in pyochelin biosynthesis, isomerizing chorismate to isochorismate (Figure 3A) [29,30]. PchA is part of the menaquinone, siderophore, and tryptophan (MST) family of enzymes that are Mg_{2+} -dependent chorismate utilizing enzymes [34–36]. Although the structure of PchA remains unsolved, the structure is hypothesized to be homologous to the isochorismate synthases, EntC [37,38] and MenF [35] (both *E. coli* enzymes) and the salicylate synthases Irp9 [39**] and MbtI [34,40,41] (Figure 3B). Interestingly, Irp9 and MbtI are the initiating enzymes to generate salicylate-capped siderophores for *Yersinia spp.* and *Mycobacterium tuberculosis*. These enzymes generate salicylate, first performing the isomerase activity of PchA and then the lyase activity, described below for PchB, in a single active site. The active site amino acids for all four isochorismate and salicylate synthase enzymes are highly conserved [42], catalyzing the isomerase activity using a general acid – general base mechanism [43]. The active site is found at the interface of two structural domains capped shut by the binding of a catalytically required magnesium ion, preventing substrates entering or products leaving the active site. Magnesium ions at high concentrations are found to be inhibitory for isochorismate synthase enzymes, but promote lyase activity in the salicylate synthase enzymes [39**]. Interestingly, ferrous ions bind to the catalytic metal binding site with nanomolar affinity for both isochorismate and salicylate synthase enzymes inhibiting catalytic turnover and suggesting a potentially physiologically relevant negative feedback mechanism for siderophore production [39**].

PchB, isochorismate-pyruvate lyase

PchB is the second enzyme in the pyochelin biosynthetic pathway, cleaving isochorismate to salicylate and pyruvate, a lyase activity (Figure 3C) [30]. PchB has no structural homology to the salicylate synthase enzymes that perform the lyase activity (described above), and is instead a structural homologue of *E. coli* chorismate mutase (EcCM) of the AroQ class (Figure 3D). Interestingly, PchB has adventitious chorismate mutase activity, albeit with much lower catalytic efficiency. The chorismate mutase reaction is a pericyclic Claisen rearrangement converting chorismate to prephenate [44,45]. The mechanism of the lyase reaction is an asynchronous and sigmatropic pericyclic reaction, with a quantitative hydrogen transfer from the ring to the pyruvylenol tail, substantiated by NMR experiments [46] and further supported computationally [47]. While this mechanism has also been proposed for the lyase-active salicylate synthases, [48] a second hypothesis for the salicylate synthases is that the reaction is performed by acid-base chemistry [49]. PchB is as an intertwined dimer, in which each monomer consists of three helices [44,50,51]. The active site loop is between helix 1 and helix 2 and is fully ordered when products are bound, whereas in the apo-structure the loop is disordered suggesting an open state for substrate entry or product egress, with closure during catalysis [50]. Lys42 is part of the mobile site loop and is

hydrogen bonded to the pyruvate product [44]. Mutating Lys42 to an Ala lowers lyase activity 100-fold although active site architecture is maintained suggesting that the chemical nature of Lys42 is key for efficient catalysis [44].

Stuffed tailoring domains in pyochelin biosynthesis: PchE and PchF

Most commonly, tailoring domains are incorporated into an NRPS module as an independent domain following the P-domain. However, in some cases tailoring domains are inserted within the A-domain, considered “interrupted” adenylation domains [52]. The tailoring domains of both PchE and PchF are stuffed into interrupted adenylation domains between the core sequence motifs, A8 and A9, of the A_{sub}-domain (Figure 4A) [33,52]. The adenylation-tailoring didomains lack structural characterization and have limited mechanistic data [53,54].

Epimerase domain of PchE

PchE incorporates the first L-cysteine into the growing chain, which is cyclized and epimerized to a D-thiazoline in the final pyochelin product. The condensation domain is specialized, also being called a cyclization domain, because it cyclizes the cysteine to make the thiazoline ring. Cyclization domains are common in NRPS assembly lines generating natural products with thiazoline or oxazoline derivatives [9,55]. The substrate for the epimerase domain is not a free cysteine amino acid, but is instead the product of the condensation reaction of L-cysteine with the upstream salicylic acid [32]. Experiments designed to determine if the conjugated L-cysteine was cyclized before or after epimerization were inconclusive, although the authors lean toward epimerase activity preceding cyclization (Figure 4B, **upper path**) [32]. However, this would require an additional domain movement. If the cysteine is epimerized before cyclization, the peptide bond would be formed in the condensation domain, epimerized in the stuffed tailoring domain, and then move back to the condensation domain for cyclization. The peptide would only have to visit the condensation domain once if both condensation and cyclization occur concurrently or sequentially, before epimerization (Figure 4B, **lower path**).

Currently there is no determined structure for any stuffed-epimerase domains, but structures of the independent epimerase domain from TycA (A-P-E) [56] of tyrocidine biosynthesis and an apo- and holo-P-epimerase didomain structure from GrsA (A-P-E) [57*] of gramicidin S biosynthesis have been determined, both from *Bacillus brevis*. The canonical epimerase domains of TycA and GrsA have similar active site motifs and share secondary structure homology to C-domains [58]. However, the epimerase domain of PchE (PchE-E) does not share any sequence similarity with TycA or GrsA epimerase domains and is considered a noncanonical epimerase domain [32].

Methyltransferase domain of PchF

PchF adds the second L-cysteine to the growing peptide chain. After condensation to the upstream peptide and cyclization, the thiazoline is reduced to thiazolidine by NADPH-dependent PchG, described below. The stuffed methyltransferase domain (M-domain) of PchF catalyzes an S-adenosyl methionine (SAM)-dependent methyl transfer to the nitrogen

of the thiazolidine ring (Figure 4C) [31,33]. The M-domain is a Class I methyltransferase possessing the consensus (GxG) SAM-binding motif [59]. MtfA, a SAM-dependent *N*-methyltransferase accessory protein of vancomycin biosynthesis from *Amycolatopsis orientalis*, shares a 32% sequence identity with the M-domain of PchF. MtfA transfers the SAM methyl group to the backbone nitrogen of leucine in the vancomycin-type antibiotics [60]. Grouped into five different classes, methyltransferases exhibit diverse tertiary structure and different binding modes of the cofactor and substrate. SAM, the methyltransferase product *S*-adenosylhomocysteine (SAH), and the inhibitor sinefungin were separately crystallized with MtfA (Figure 4D) [60]. SAM and SAH bound in the consensus SAM-binding motif of the Rossmann fold, corresponding to binding modes seen in other Class I SAM-dependent methyltransferases [60].

Stuffed epimerase as a defunct methyltransferase

Interestingly, the epimerase domain of PchE and the methyltransferase domain of PchF share ~21% sequence identity and ~55% sequence similarity, suggesting the epimerase domain of PchE is more similar to a dysfunctional SAM-dependent M-domain than a canonical epimerase domain. This can be rationalized mechanistically, since the first step of the methyltransferase activity is removal of a proton to generate an enol that is randomly reprotonated in the absence of SAM, racemizing the position. Only the appropriate epimer is a substrate for subsequent steps. Indeed, the PchE-epimerase possesses a Gly to Arg mutation in the conserved SAM-binding motif (RxG), suggesting interruption of SAM binding [32,52]. This hypothesis can be applied more broadly to other pyochelin-like natural products. The biosynthesis of enantio-pyochelin (Figure 4E), the enantiomer of pyochelin generated by *Pseudomonas fluorescens* under iron-limiting conditions, lacks the stuffed epimerase domain [61]. A stuffed epimerase of the multi-module NRPS called high molecular weight protein 2 (HMWP2) in yersiniabactin biosynthesis may also be a defunct methyltransferase, sharing the same substrate as PchE and possessing a Gly to Arg mutation in the SAM-binding motif (GxRxG) [62]. Watasemycin, an NRPS natural product from *Streptomyces venezuelae*, possesses a stuffed methyltransferase domain with neither methyltransferase nor epimerase activity, though it has the same domain architecture and substrates as PchE [63]. Further structure-function studies of the aforementioned enzymes will be necessary to elucidate the differences between the methyltransferase, epimerase, and nonfunctional stuffed domains of pyochelin-like natural products. Stuffed domains have also been with other, unrelated catalytic activities, such as the oxidase activity of epothilone biosynthesis [64] and the monooxygenase activity of myxothiozole [65] and thuggacin [66] biosynthesis.

Stand-alone tailoring domain: PchG

The terminal cyclized cysteine must be reduced to a thiazolidine while tethered to the Ppant tail on PchF, prior to final methylation and release of the mature pyochelin by the thioesterase domain (Figure 4F) [27]. PchG, a stand-alone tailoring enzyme, is proposed to perform the reduction by proton donation from a general acid and subsequent hydride transfer from NADPH. Structures of a functionally homologous enzyme of yersiniabactin biosynthesis [67], Irp3, have been determined: an apo-form, NADP₊-bound form, and with

NADP⁺- and substrate analog-bound (Figure 4G) [68*,69]. Irp3 is structurally similar to sugar oxidoreductases and to biliverdin reductase and possesses an N-terminal Rossmann fold domain for binding NADPH cofactor and a C-terminal α/β domain for substrate binding and dimerization [69]. Upon binding of a substrate analogue, a long loop creates a lid over the active site of the opposing monomer leaving a tunnel that is the proposed entrance of the Ppant tether [68*]. As the authors point out, the binding of the substrate analog observed in this study may not be physiologically relevant, because the substrate analog binds in a position that would promote incorrect stereochemistry if hydride transfer were to take place [68*].

Conclusion

Siderophores are small, iron-chelating molecules generated by NRPS systems. NRPS structures have provided insight into how these nanomachines form peptide bonds outside of the ribosome. Recently, several full-module structures have elucidated the different domain interactions and domain alternations that occur during peptide assembly. Additional full-module and cross-module structures are necessary to understand domain interactions between modules. While NRPS enzymes generate peptide assembly, accessory and tailoring enzymes provide the chemical diversity that make each natural product unique. Using pyochelin biosynthesis as a model system, we have provided examples of the differing accessory and tailoring activities required to generate even a small natural product. In pyochelin biosynthesis, accessory proteins are required to make the salicylate that caps the N-terminal end. Tailoring domains in the generation of pyochelin fall into two categories. Epimerase and methyltransferase domains are stuffed within the A_{sub}-domain. This understudied class of tailoring domains may provide a unique assembly line logic. Pyochelin biosynthesis also requires a stand-alone tailoring reductase. While NRPS enzymes have been the subject of considerable investigation and the field is considered mature, there are still many unanswered questions regarding peptide bond formation and the decoration of these peptides to generate the chemical structures that provide biodiversity.

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Highlights

- Domain interactions and alternations of full- and cross-module NRPSs are discussed.
- Biodiversity of NRPS products is provided by accessory and tailoring enzymes.
- Stuffed tailoring domains are an understudied class of NRPS biosynthesis.
- Stuffed epimerase and methyltransferase domains share architecture and substrates.

Figure 1. NRPS assembly line and crystal structures highlighting intra- and inter- domain contacts and movements

A) NRPS natural product biosynthesis takes place in three phases: initiation, condensation, and termination. Adenylation and subsequent thiolation of the amino acid onto the Ppant tether prime a module. A condensation reaction is performed between amino acids tethered to primed modules to generate a peptide bond, thereby elongating the peptide chain. Termination and release of the mature peptide occurs by hydrolysis within a terminal thioesterase domain after transfer of the product to a conserved serine. Throughout this review: yellow = adenylation (A); blue = peptidyl carrier domain (P); green = condensation (C); red = thioesterase (T). **B)** The first crystal structure of a complete NRPS module, SrfA-

C (PDB: 2VSQ). Note that the A_{core}-domains (light yellow) are aligned in parts B thru F of this figure to highlight domain movements. The substrate leucine (cyan spheres) is in the A_{core}-domain. The A_{sub}-domains are in dark yellow. **C)** The full module of AB3403 (PDB: 4ZZI) with the Ppant tether (purple carbon and red oxygen spheres) in the closed C-domain ready to accept an upstream peptide. The substrates AMP and Gly (cyan spheres) are in the A_{core}-domain active site. **D)** A full-module crystal structure of EntF with its MLP, YbdZ (black), bound to the A_{core} (PDB: 5JA1). The mechanistic-based inhibitor, Ser-AVS, is covalently attached to the Ppant tether (spheres) which is trapped in the A_{core}-domain. **E)** Domain movements of the A_{sub}- and P-domain are highlighted by the multi-domain structures of LgrA. These snap-shots show the movement of the A_{sub}-domain in “open” and “closed” (PDB: 5ES5) conformations during adenylation, and the movement of the A_{sub}- and P-domains during thioester formation (Val-NH-Ppant analog shown in spheres) (PDB: 5ES8) and for tailoring by the formyltransferase domain (pink) (PDB: 5ES9). **F)** The cross-module structure of DhbF containing an A- and P-domain from module 1 and C-domain from module 2. The cartoon emphasizes the cross-module construct. The MLP from *Geobacillus sp.* is bound to the A_{core}-domain and shown in black. The mechanistic-based inhibitor, Gly-AVS (spheres), is covalently bound to the Ppant arm in the A_{core}-domain (PDB: 5U89).

Figure 2. Pyochelin biosynthesis

A) Three NRPS enzymes consisting of eleven different domains, two accessory enzymes, and one stand-alone tailoring enzyme are used to generate pyochelin, a siderophore from *P. aeruginosa*. Domain abbreviations are as in Figure 1, with the exception that domain types are numbered in the assembly line (A1 = first adenylation domain), and these additions: ICS = isochorismate synthase, IPL = isochorismate-pyruvate lyase, R = reductase. **B)** Biosynthesis is initiated by PchA and PchB, which generate the hydroxy acid, salicylate, for the N-terminal cap. PchA converts chorismate to isochorismate and PchB converts isochorismate to salicylate and pyruvate. Salicylate is activated by PchD and delivered to the

Ppant tether of the first P-domain in PchE. Adenylation and thiolation of L-cysteine occur in the second module. Condensation with the upstream salicylate and cyclization and epimerization of L-cysteine to D-thiazoline occurs in the elongation module of PchE. The termination module in PchF, primed with an L-cysteine, condenses and cyclizes the cysteine with the upstream peptide chain to form a hydroxyphenyl-bis-thiazoline intermediate. PchG, the stand-alone tailoring domain, reduces the second thiazoline to a thiazolidine in an NADPH-dependent manner. Subsequent SAM-dependent methylation of the thiazolidine nitrogen is catalyzed by the stuffed tailoring methyltransferase domain of PchF. Mature pyochelin is hydrolyzed and released by the T-domain.

Figure 3. Accessory enzymes in pyochelin biosynthesis

A) PchA is an isochorismate synthase (ICS) converting chorismate to isochorismate. **B)**

PchA structural homolog MenF in the "open" (PDB: 3BZM) and "closed" (PDB: 3BZN) structures, showing the magnesium ion (green) that creates a button closing the active site.

C) PchB is an isochorismate pyruvate-lyase (IPL) converting isochorismate to salicylate and pyruvate. **D)** Crystal structure of dimeric PchB (PDB: 3REM). Monomers are shown in blue and gray, with the products (green sticks) in the active sites.

Figure 4. Tailoring enzymes

A) The A-domain of AB3403 (PDB: 4ZXI) highlighting the core adenylation sequences A8 and A9 (purple), between which tailoring domains are stuffed. Also highlighted is a sequence-based prediction of where the PchE epimerase-domain (blue) and PchF M-domain (green) are stuffed into the A_{sub}-domain. **B)** PchE first condenses the upstream salicylate to the L-cysteine within its condensation domain. From here, two hypotheses arise. The first (top) suggests that the peptide tether moves to the epimerase domain to epimerize the L-cysteine to D-cysteine, and then moves back to the condensation/cyclization domain to cyclize the D-cysteine to D-thiazoline. The second hypothesis (bottom) suggests that after

condensation to the upstream salicylate, L-cysteine cyclizes to L-thiazoline while in the condensation domain and then moves to the epimerization domain to generate D-thiazoline, resulting in less domain movement. **C)** The “stuffed” methyltransferase domain of PchF performs a SAM-dependent methyl transfer to the nitrogen of the thiazolidine ring. **D)** MtfA (PDB: 3G2O), a stand-alone SAM-dependent nitrogen methyltransferase of vancomycin biosynthesis from *Amycolatopsis orientalis*, with SAM (cyan sticks) bound. **E)** Pyochelin-like natural products made by NRPSs: Pyochelin – *Pseudomonas aeruginosa*. Enantio-pyochelin – *Pseudomonas fluorescens*. Yersiniabactin – *Yersinia pestis*. Watasemycin – *Streptomyces venezuelae*. **F)** PchG, the stand-alone tailoring enzyme, is a NADPH-dependent reductase converting the second thiazoline of the PchF intermediate to a thiazolidine. **G)** Irp3 (PDB: 5KVS), the PchG homologue for the production of yersiniabactin, is dimeric. The M-N loop closes the active site of an opposing monomer, with NADP₊ (green sticks) and substrate analog bound (cyan sticks).