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Acyl peptidic siderophores: Structures, biosyntheses and post-assembly modifications

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Abstract:

Acyl peptidic siderophores are produced by a variety of bacteria and possess unique amphiphilic properties. Amphiphilic siderophores are generally produced in a suite where the iron(III)-binding headgroup remains constant while the fatty acid appendage varies by length and functionality. Acyl peptidic siderophores are commonly synthesized by non-ribosomal peptide synthetases (NRPS); however, the method of peptide acylation during biosynthesis can vary between siderophores. Following biosynthesis, acyl siderophores can be further modified enzymatically to produce a more hydrophilic compound, which retains its ferric chelating abilities as demonstrated by pyoverdine from *Pseudomonas aeruginosa* and the marinobactins from certain *Marinobacter* species. Siderophore hydrophobicity can also be altered through photolysis of the ferric complex of certain β-hydroxyaspartic acid-containing acyl peptidic siderophores.

Keywords

Amphiphilic siderophore, biosynthesis, acyl peptide, post-assembly modification

1. Introduction:

In response to the low concentration of soluble iron in bacterial growth environments, many bacteria produce siderophores, low molecular weight, ferric iron chelators to solubilize and transport Fe(III) into the cell. The overall structure of siderophores can vary among species; however, the functional groups that chelate ferric iron are relatively conserved among catecholate, hydroxamate and α-hydroxycarboxylate groups (Sandy and Butler 2009). Based on comparatively conserved pathways for biosynthesis of catecholate and hydroxamate functional groups, genome mining is becoming a useful tool to predict the types of siderophores produced by a bacterial species, particularly in conjunction with the proximity of these biosynthetic genes to well-characterized iron uptake and transport genes (Crosa and Walsh 2002; Challis et al. 2000; Walsh et al. 2001).

Many marine siderophores are distinguished by the presence of a fatty acid appendage connected to a peptidic or citrate-based headgroup resulting in an amphiphilic compound (Martinez et al. 2000; Homann et al. 2009; Martin et al. 2006; Zane et al. 2014). Amphiphilic siderophores are generally produced in a suite where the headgroup, which coordinates Fe(III) remains constant, but the fatty acid appendage ranges in length, degree of unsaturation and functionality. Little is known about how the fatty acids benefit siderophore-mediated iron uptake or how the amphiphiles interact with the bacterial membrane; however, it is becoming evident that this structural characteristic is more common among *both* marine and non-marine siderophores than previously recognized. This review will cover the structure, biosynthesis and post-assembly modification of acyl peptidic siderophores. The class of citrate-derived acylated siderophores will not be reviewed here.

2. Structures of amphiphilic siderophores

2.1 Amphiphilic siderophores from marine environments

Acyl peptidic siderophores from marine bacteria span a wide range across the amphiphilic spectrum, from those that are hydrophobic and remain cell-associated to those that are hydrophilic. Acyl siderophores with short peptides (4 amino acids) and longer fatty acid tails (≥ C16), such as the amphibactins (Figure 1), are quite hydrophobic and usually remain cell-associated, even after centrifugation of the bacterial culture. The amphibactins have been isolated from multiple species of marine bacteria, including the well-studied hydrocarbon degrader, *Alcanivorax borkumensis* SK2 (Martinez et al. 2000; Vraspir et al. 2011; Kem et al. 2014). Some amphibactin-producing species, however, make predominantly shorter fatty acid derivatives that can be isolated from culture supernatants (Vraspir et al. 2011). The moanachelins, produced by *Vibrio* sp. Nt1, are structurally related to the amphibactins but differ by

one amino acid where the internal serine of the amphibactins is replaced with either a glycine or an alanine (Gauglitz and Butler 2013). The moanachelins are the first example of amino acid variability in a suite of amphiphilic siderophores produced by a single bacterium, although the origin of this variability is not yet known.

Acyl siderophores with longer peptidic headgroups, such as the loihichelins (8 amino acids) and aquachelins (7 amino acids) (Figure 1), are quite hydrophilic and are isolated from the supernatant of harvested cultures (Martinez et al. 2000; Homann et al. 2009). The marinobactins (Figure 1) produced by *Marinobacter sp*. DS40M6 have a headgroup consisting of six amino acids appended by a C12-C18 fatty acid and are found partitioned between both the supernatant and the cell pellet (Martinez et al. 2000; Martinez and Butler 2007).

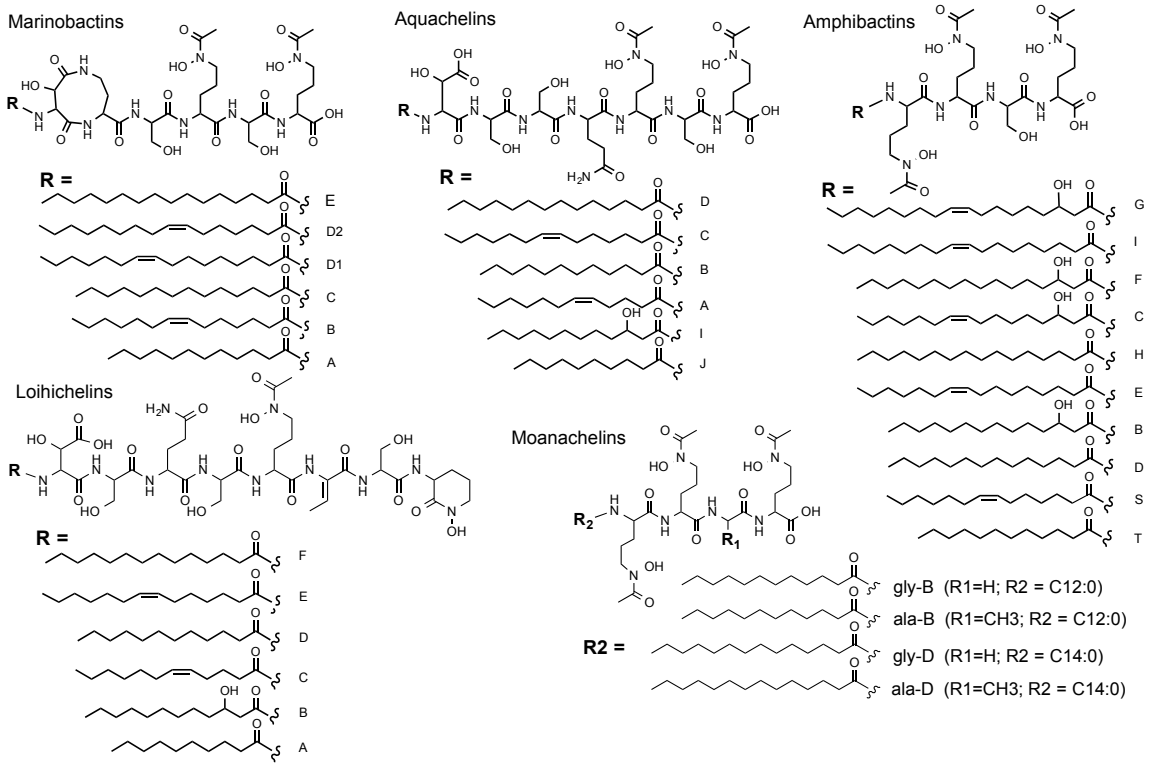

Figure 1. Structures of peptidic acyl siderophores produced by different marine bacteria isolated from ocean surface waters. Moanachelins gly-C (R1=H; R2=C14:1) and gly-E (R1=H; R2=C16:1) were also isolated, however, the location of the double bond has not been determined.

Recently, the amphi-enterobactins, which are fatty acid derivatives of the well-known siderophore enterobactin (Figure 2), were isolated from the marine bioluminescent bacterium, *Vibrio harveyi* BAA-1116 (reclassified as *Vibrio campbellii*) (Zane et al. 2014). Unlike enterobactin, the amphi-enterobactins have a fourth serine incorporated into the trilactone backbone forming a tetralactone ring. This additional serine is used as the attachment point for a fatty acid appendage in place of a 2,3-dihydroxybenzoic acid moiety. Due to the already hydrophobic nature of

enterobactin (Luo et al. 2006), the addition of the acyl moiety to the amphi-enterobactins greatly increases their hydrophobicity and causes the siderophores to associate with the cell (Zane et al. 2014).

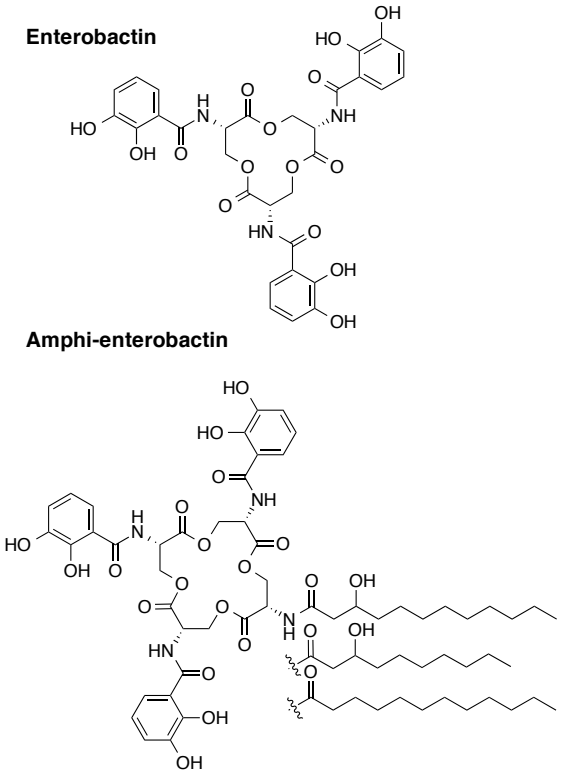

Figure 2. The amphi-enterobactins produced by *Vibrio harveyi* BAA-1116 are acylated derivatives of the prototypic siderophore, enterobactin. Amphi-enterobactins with a C12:1, C12:1-OH, C14:1, and C14:1-OH fatty acid were also isolated, however, the location of the double bond was not determined.

2.2 Amphiphilic siderophores from pathogens and non-marine environmental isolates

Amphiphilic siderophores are not limited to bacteria in the marine environment. The mycobactins are acyl peptidic siderophores produced by *Mycobacteria* species and differ slightly in the peptidic core structure depending on the producing species (Ratledge 2004; Snow 1965). The human pathogenic bacterium, *Mycobacterium tuberculosis*, produces mycobactin T along with the more hydrophilic mycobactin derivative, carboxymycobactin T (Figure 3). Unlike the acyl siderophores previously discussed, the internal lysine of the mycobactin core is acylated with a C18-C23 fatty acid rather than the N-terminal residue (Ratledge 2004). Additionally, the N^6 of this lysine is hydroxylated forming one of the hydroxamate groups, which coordinates Fe(III).

The carboxymycobactins are mainly produced by pathogenic species of *Mycobacteria* and differ slightly in structure from the mycobactins due to a shorter fatty acid appendage, which is also functionalized with a terminal carboxylic acid or methyl ester (Gobin et al. 1995; Lane et al. 1995). The shorter, functionalized tail of the

carboxymycobactins allows them to be excreted into the extracellular milieu. It is hypothesized that the carboxymycobactins transfer iron to the mycobactins in the bacterial membrane, which act as short-term storage molecules for Fe(III) (Gobin and Horwitz 1996).

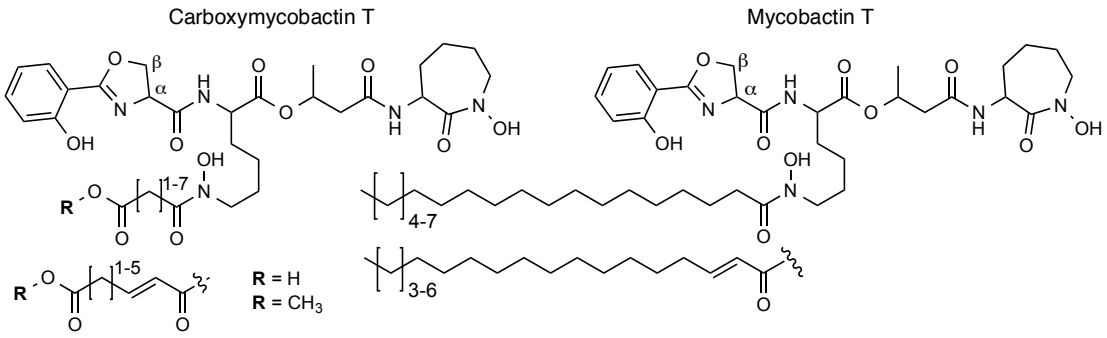

Figure 3. Siderophores produced by *Mycobacterium tuberculosis*. The α- and β- labeled carbons on the oxazoline ring of mycobactin T and carboxymycobactin T may be methylated.

Cupriachelin and taiwachelin are structurally similar amphiphilic siderophores isolated from different *Cupriavidus* species, which are often found in soil and freshwater environments (Figure 4) (Kreutzer et al. 2012; Kreutzer and Nett 2012). Both siderophores have β-hydroxyaspartic acid residues in their headgroup providing unique photochemical properties, which will be discussed later in this review. The serobactins, isolated from the plant endophytic bacterium, *Herbaspirillum seropedicae* Z67, are structurally similar to cupriachelin and taiwachelin (Figure 4) (Rosconi et al. 2013). All three siderophores are quite hydrophilic and were isolated from culture supernatants.

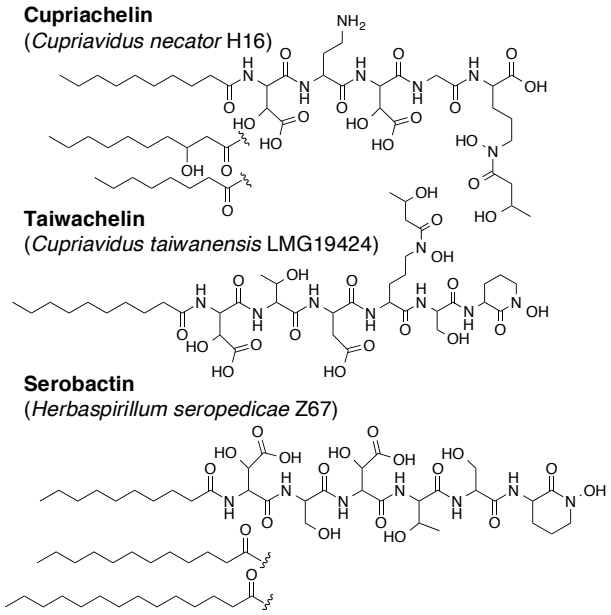

Figure 4. Non-marine acyl siderophores containing β-hydroxyaspartic acid residues.

3. Biosynthesis of amphiphilic siderophores

3.1 General NRPS and hybrid NRPS-PKS-mediated biosynthesis

Siderophores are often comprised of a combination of proteogenic and non-proteogenic amino acids as a result of being synthesized by large, assembly line-like proteins called non-ribosomal peptide synthetases (NRPS). A basic NRPS module consists of an adenylation, condensation and thiolation domain. The adenylation domain selects a specific amino acid from a pool of substrates and activates the amino acid to form the corresponding aminoacyl adenylate through the consumption of ATP (Figure 5A) (Stachelhaus et al. 1999). The aminoacyl adenylate is then loaded onto an adjacent thiolation domain (also referred to as the peptidyl carrier protein, PCP) where it is stabilized through the formation of a thioester bond with a phosphopantetheinyl (Ppant) prosthetic group (Figure 5B). Prior to binding the aminoacyl adenylate, the apo-PCP is converted to its holo form through the post-translational transfer of a 4'-phosphopantetheinyl (4'-PP) cofactor of coenzyme A onto a serine residue of the enzyme active site to generate the Ppant prosthetic group. NRPSs can also include various modifying domains, such as, epimerization, N- or C-methylation, or cyclization, which increase the structural diversity and biological activity of non-ribosomally synthesized natural products (Marahiel et al. 1997). In a Type A NRPS, the peptidyl chain grows unilaterally as the condensation domains catalyze peptide bond formation between the incoming amino acid and the growing peptide chain on adjacent thiolation domains. Type B NRPSs work iteratively by utilizing the modules and domains more

than once to generate small peptides of repeating sequences. Non-linear NRPSs (Type C) produce natural products whose structures do not correspond to the linear arrangement of the NRPS modules and domains (Grunewald and Marahiel 2006). Release and termination of the peptide chain is commonly performed through hydrolysis or cyclization at the thioesterase domain (Figure 5).

Adenylation domains have high substrate specificity, which allow specific amino acids to be incorporated into the growing peptide chain. Overall, adenylation domains have a low sequence similarity to one another but they do contain core motifs (A1-A10) of high homology (Stachelhaus et al. 1999). The substrate specificity of adenlylation domains is mediated by a signature sequence consisting of eight amino acids lining the substrate-binding pocket (Stachelhaus et al. 1999; Challis et al. 2000). These signature sequences form a degenerate NRPS code where one or more codes can be utilized for the same amino acid. The signature sequences can be used to predict which amino acid will be incorporated into a growing peptidyl chain (Stachelhaus et al. 1999).

Polyketide synthetases (PKSs) are also important components of the biosynthesis of some siderophores. Type I PKSs, are arranged in a modular fashion, and each module adds a new carbon unit to the growing polyketide chain (Shen 2003; Staunton and Weissman 2001). Type I PKSs are commonly composed of ketosynthase (KS), acyltransferase (AT) and acyl carrier protein (ACP) domains (Shen 2003). Some siderophores, such as the mycobactins, are synthesized by hybrid NRPS-PKS systems as described below.

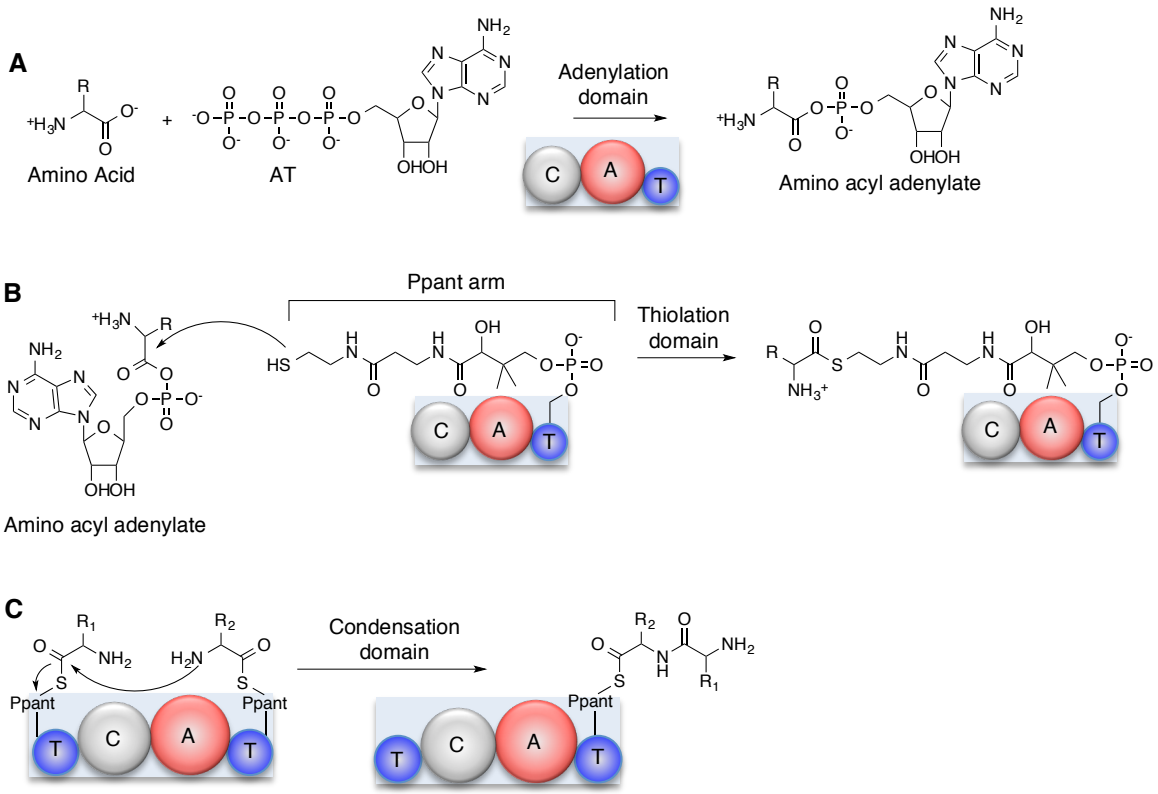

Figure 5. General NRPS biosynthetic scheme. (A) The adenylation domain selects a specific amino acid substrate and converts it to an aminoacyl adenylate under the consumption of ATP (B) The activated aminoacyl adenylate is then transferred to the Ppant arm of the thiolation domain (C) The condensation domain catalyzes amide bond formation of amino acids on adjacent thiolation domains. C = condensation domain; A = adenylation domain; T = thiolation domain; Ppant = phosphopantetheinyl.

3.2 Biosynthesis of the amphi-enterobactins

The amphi-enterobactins are comprised of three 2,3-dihydroxybenzoyl-L-serine units and one L-serine-fatty acid unit in a tetralactone ring. The proposed biosynthetic pathway of the amphi-enterobactins (**Figure 6**) contains six genes (AebA-F), which are predicted homologues of the well-studied enterobactin biosynthetic genes (Zane et al. 2014). AebA, AebB (N-terminal domain) and AebC convert chorismate to 2,3-dihydroxybenzoic acid. AebB (C-terminal domain), AebE, AebD and AebF are NRPS enzymes, which polymerize and cyclize the L-Ser-dihydroxybenzoic acid and L-Ser-fatty acid. Unlike EntF, which just catalyzes the condensation of L-serine and dihydroxybenzoic acid during enterobactin biosynthesis (DHBA) (Gehring et al. 1998), the condensation domain of AebF displays unique dual activity by catalyzing the amide bond formation between L-ser and DHBA *and* the L-ser and the fatty acid. To initiate biosynthesis, the incorporated fatty acid is activated by the fatty acyl CoA ligase (FACL), AebG, which is in close proximity to AebA-F. A knockout mutant of *aebG* completely abolishes amphi-

entrobactin production indicating that *aebG* is necessary for the acylation and initiation of amphi-enterobactin biosysnthesis (Zane et al. 2014).

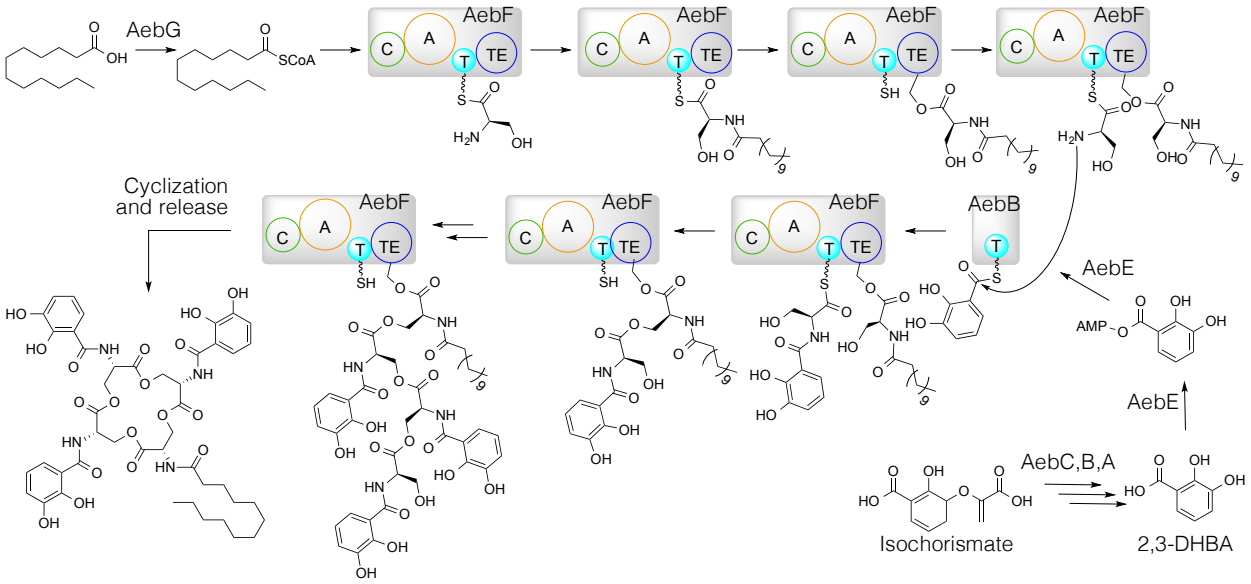

Figure 6. Proposed biosynthetic pathway for the amphi-enterobactins (Zane et al. 2014). Biosynthesis begins with the activation of a fatty acid by the fatty acyl CoA ligase, AebG. The fatty acid ranges from a C10-C14 and varies in degree of unsaturation and hydroxylation. C, condensation domain; A, adenylation domain; T, thiolation domain; TE, thioesterase domain. Reprinted with permission from Zane, H.; Naka, H.; Rosconi, F.; Sandy, M.; Haygood, M.; Butler, A. *Journal of the American Chemical Society* 2014, 136 (15), 5615-5618. Copyright 2014 American Chemical Society.

3.3 Mixed NRPS-PKS biosynthesis: Mycobactins

The gene cluster encoding the biosynthetic enzymes for mycobactin assembly was discovered by the research group of Christopher Walsh following the sequencing of the *M. tuberculosis* genome in 1998 (Quadri et al. 1998); however, until recently, the genes responsible for the acylation of the mycobactins were not known. The mycobactins and carboxymycobactins are synthesized by hybrid NRPS-PKS proteins encoded by two gene clusters designated as *mbt-1* and *mbt-2* (Figure 7). The mycobactin core is assembled by *mbt-1*-encoded proteins, which are comprised of both NRPSs (MbtA, MbtB, MbtE, MbtF) and PKSs (MbtC, MbtD) (Quadri et al. 1998; McMahon et al. 2012). The adenylation domain of MbtB, which incorporates L-serine or L-threonine to form the oxazoline ring, shows specificity for threonine; however, the majority of mycobactins lack a β-methyl group, suggesting the methyl group is removed by an as yet to be determined mechanism (McMahon et al. 2012).

Gene cluster *mbt-2* consists of four open reading frames (*mbtL*, *mbtM*, *mbtN*, *mbtK*) that are necessary for mycobactin biosynthesis. The *mbt-2*-encoded proteins transfer an acyl substituent onto the side chain of a lysine

residue for incorporation into the mycobactin core (Krithika et al. 2006; Vergnolle et al. 2013; McMahon et al. 2012). However, there is some debate about whether the lysine is hydroxylated by MbtG prior to or following its acylation. Using a lipidomics profiling platform, Moody and co-workers (Madigan et al. 2012) isolated dideoxymycobactins from Δ*mbtG M. tuberculosis*, which suggests that the mycobactins are hydroxylated following their acylation and incorporation into the peptide backbone. MbtM is homologous to fatty acyl-AMP ligases (FAAL) and activates fatty acids into their corresponding acyl adenylates under the consumption of ATP. The activated fatty acid is transferred to the Ppant arm of the carrier protein, MbtL, and MbtK transfers the fatty acid onto the ε-amino group of lysine. (Figure 7). MbtM prefers long-chain fatty acids and does not bind short acyl chains or dicarboxylic acid substrates suggesting that another protein is expressed to incorporate or functionalize the carboxymycobactin fatty acid tail (Vergnolle et al. 2013). A point of unsaturation is introduced at the α-β position of the fatty acid by the acyl-ACP dehydrogenase, MbtN, which catalyzes the dehydrogenation of the ACP-bound substrate (Krithika et al. 2006). Understanding the biosynthetic machinery for mycobactin assembly has led to a new strategy for antibiotic development, which targets siderophore biosynthesis (Ferreras et al. 2005; Somu et al. 2006; Neres et al. 2008; Cisar and Tan 2008; Engelhart and Aldrich 2013).

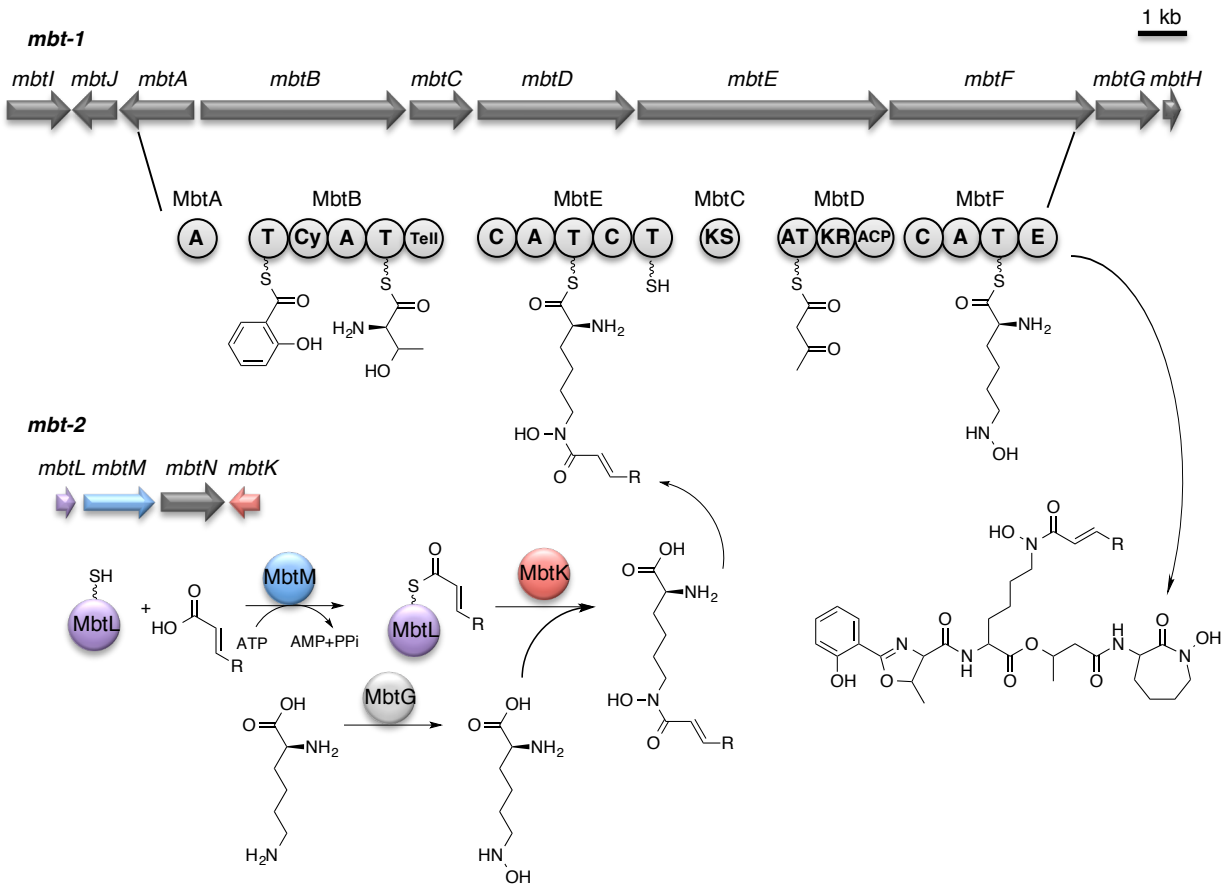

Figure 7. Proposed biosynthetic scheme for the mycobactins from *Mycobacterium tuberculosis*. The *mbt-2*-encoded proteins transfer an acyl substituent onto a N-hydroxy-lysine side chain for incorporation into the mycobactin core, which is assembled by *mbt-1*-encoded proteins. Only the α, β unsaturated fatty acids are shown (R = 15-18 carbons), although saturated fatty acids (C18-C23) are also observed. A, adenylation domain; T, thiolation domain; Cy, cyclization domain; TeII, type II thioesterase; C, condensation domain; KS, ketosynthase; AT, acyltransferase; KR, ketoreductase; ACP, acyl carrier protein; E, epimerase.

3.3.1 Developing inhibitors based on mycobactin biosynthesis

3.4 Biosynthesis of Pyoverdine

The peptide core of pyoverdine produced by *P. aeruginosa* PAO1 is synthesized by four different NRPS proteins: PvdL, PvdI, PvdJ, and PvdD (**Figure 8**) (Visca et al. 2007). The first module of PvdL (M1, **Figure 8**) is homologous to fatty acyl AMP-ligases, which activate a fatty acid for peptide acylation (Hannauer et al. 2012; Schalk and Guillon 2013). The activated fatty acid is then transferred to the carrier protein on M1 of PvdL where the condensation domain of module 2 (M2) from PvdL catalyzes amide bond formation between the activated fatty acid and the L-glutamic acid selected by M2. A pyrophosphate exchange assay of M1 from PvdL showed a preference

for a C14 fatty acid (Drake and Gulick 2011). The remaining PvdL modules, PvdI, PvdJ, and PvdD complete the biosynthesis of the peptidic portion of the pyoverdine core. The epimerase domains convert L-Tyr on PvdL and both L-Ser residues on PvdI to the D-stereoisomer. The periplasmic hydrolase, PvdQ, removes the fatty acid appendage from the pyoverdine precursor prior to chromophore formation by PvdP, PvdO, and PvdN to complete the biosynthesis of pyoverdine (Yeterian et al. 2010).

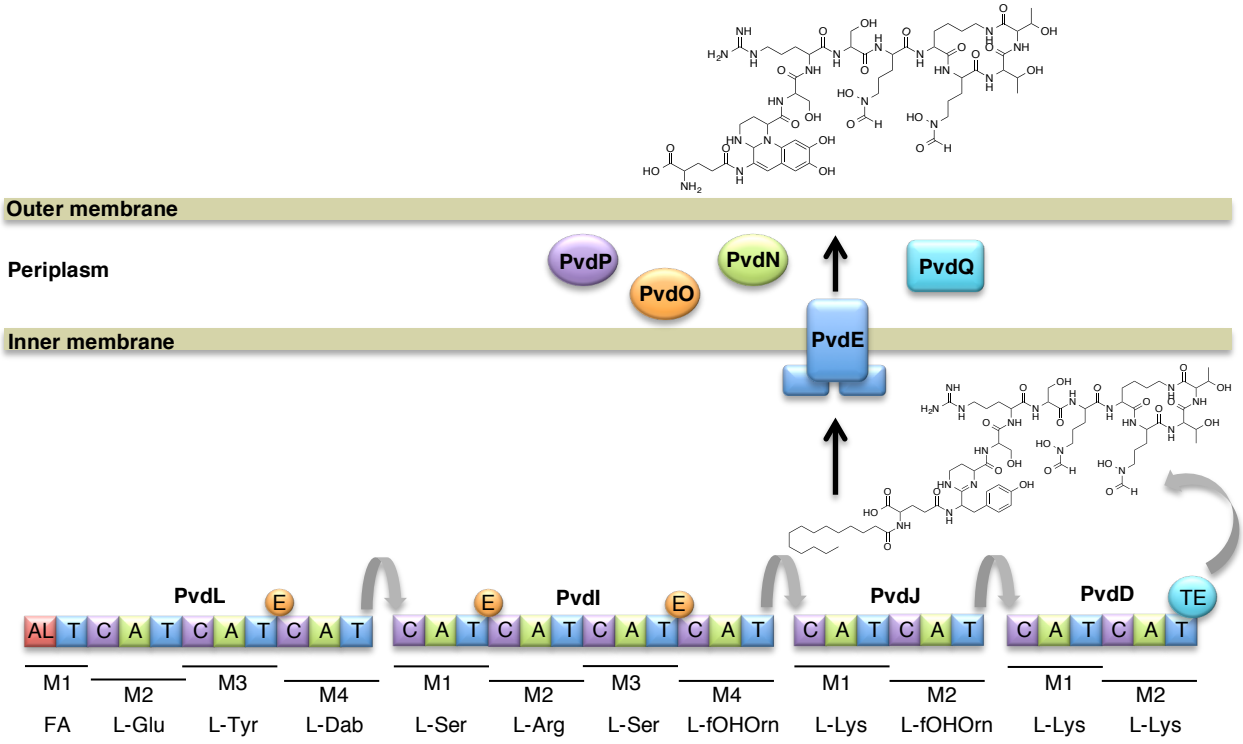

Figure 8. Proposed biosynthetic pathway of pyoverdine from *P. aeruginosa* PAO1. The starter unit of PvdL (AL) is a fatty acyl AMP-ligase that activates a fatty acid for incorporation into the peptide chain. C = condensation domain, A = adenylation domain, T = thiolation domain, TE = thioesterase domain, E = epimerase domain. Periplasmic proteins PvdP, PvdO, PvdN and PvdQ are involved in siderophore maturation prior to excretion from the cell.

3.5 Genome derived biosynthesis of amphiphilic siderophores

Genome mining has been a useful tool for the discovery of peptide natural products, including siderophores. The wealth of information regarding the biosynthesis of natural products in conjunction with the modular logic of NRPSs has enabled the structural organization of non-ribosomally synthesized siderophores to be predicted. In particular for siderophores, the sequences of proteins that synthesize common iron-binding groups (i.e. catechols and hydroxamtes) are well-conserved, as are proteins involved in siderophore biosynthesis, tailoring and transport.

3.5.1 Fatty acid activation by an external ligase

Cupriachelin is synthesized through the concerted action of four NRPS proteins: CucF, CucG, CucJ, and CucH (Figure 9A) (Kreutzer et al. 2012). CucF begins with an atypical N-terminal condensation domain suggesting it has an external ligase to activate a fatty acid for peptide acylation, similar to the biosynthesis of the amphi-enterobactins (Zane et al. 2014) (**Figure 6**) and surfactin (Kraas et al. 2010). Module 1 of CucF also contains an acyl carrier protein (ACP) domain, which tethers and transfers the activated fatty acid during biosynthesis. Module 2 of CucF ends with a TauD domain, which is predicted to catalylze the hydroxylation of the aspartate residues to form the α-hydroxycarboxylic acid moiety used in Fe(III) chelation. An analysis of the substrate specificity of the adenylation domain from CucG suggests L-Asp is hydroxylated following initiation of the adenylation step. CucH and CucJ finish the synthesis of the peptidic portion of the siderophore, as indicated in Figure 9A, however, CucH does not end in a terminal thioesterase domain. Instead, chain termination is proposed to occur by the hydrolase, CucC (Kreutzer et al. 2012).

The genome of *A. borkumensis* SK2 has two putative NRPSs predicted to be involved in amphibactin biosynthesis: ABO_2093 and ABO_2092, which are comprised of four classical NRPS modules (Figure 9B) (Kem et al. 2014). As seen with the biosynthesis of cupriachelin, module 1 of ABO_2093 begins with a N-terminal condensation domain, suggesting an external ligase activates a fatty acid for incorporation into the peptidic headgroup. Interestingly, however, no acyl carrier protein (ACP) domain is present in ABO_2093 to tether and transport the activated fatty acid. An ACP domain is also omitted in the biosynthetic gene cluster of surfactin (Kraas et al. 2010) and the amphi-enterobactins (Zane et al. 2014). Biosynthesis then appears to occur in a linear fashion, as indicated in Figure 9B, ending with a thioestease domain, which is preceded by a domain of unknown function.

3.5.2 Fatty acid activation by a fatty acyl AMP-ligase domain

The marinobactin-producing bacterium, *M. nanhaiticus* D15-8W, has two putative NRPSs predicted to be involved in siderophore biosynthesis, ENO16763 and ENO16762 (Figure 9C). The first module of ENO16763 encodes a unique domain possessing high homology to fatty acyl AMP-ligases, as seen with the biosynthesis of pyoverdine from *P. aeruginosa* PAO1 (Schalk and Guillon 2013). This module is predicted to activate a fatty acid for incorporation into the peptidic headgroup. Based on the structure of the marinobactins, the biosynthesis is proposed to occur in a linear fashion and terminates with a thioesterase domain as shown in Figure 9C. Similar to

marinobactin biosynthesis, the predicted biosynthesis of the taiwachelins (Figure 9D) (Kreutzer and Nett 2012) and serobactins (Figure 9E) (Rosconi et al. 2013) begin with a N-terminal acyl AMP-ligase starter domain, which activates a fatty acid and initiates biosynthesis.

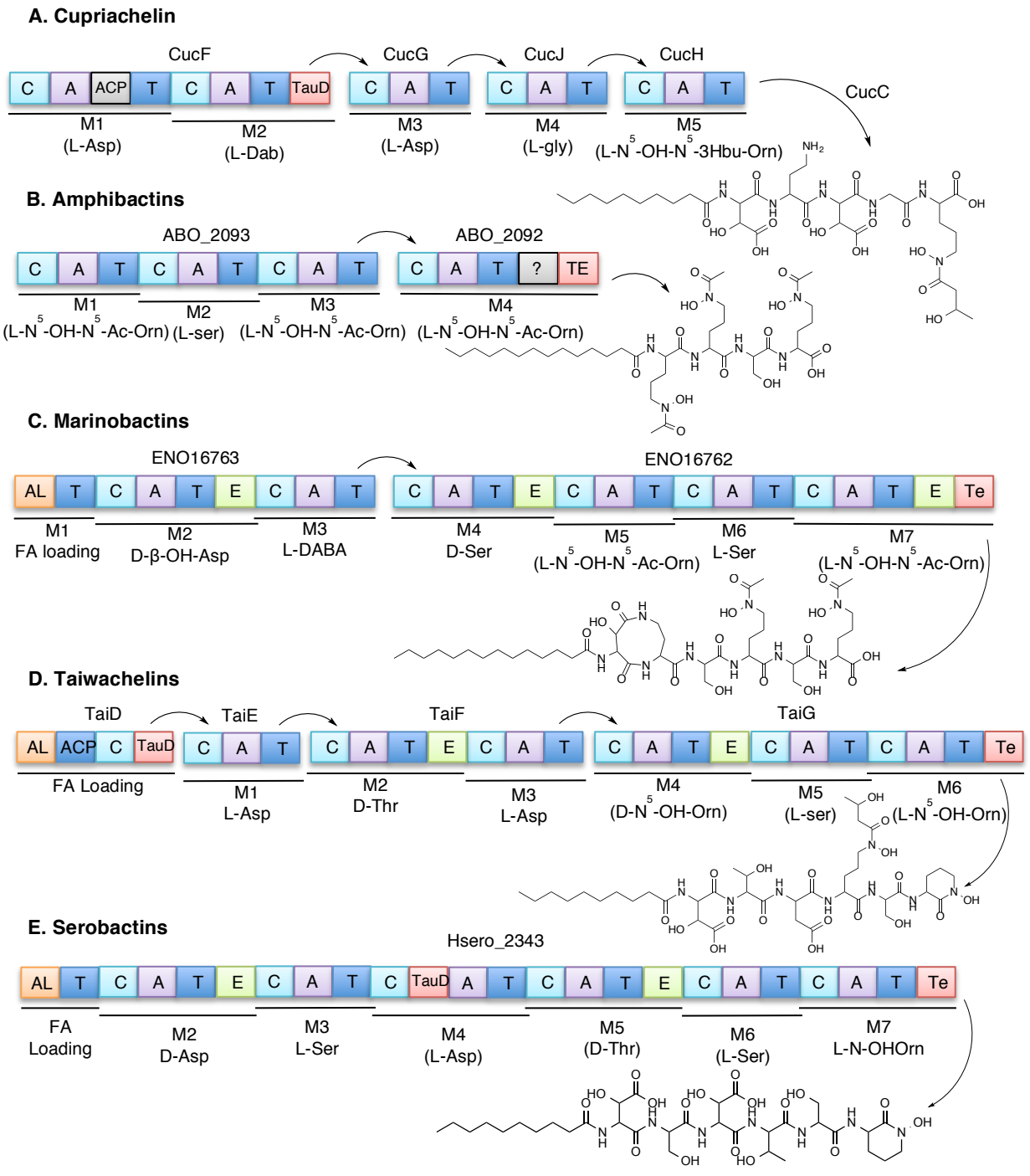

Figure 9. Proposed biosynthetic schemes for (A) Cupriachelin from *C. necator* H16 (B) the amphibactins from *A. borkumensis* SK2 (C) the marinobactins from *M. nanhaiticus* D15-8W (D) taiwachelin from *C. taiwanensis* LMG19424 (E) serobactin from *H. seropedicae* Z67 based on A domain predictions. The Asp residues from

cupriachelin, taiwachelin, and serobactin are proposed to be hydroxylated by the TauD domains following initiation of the adenylation step. C = condensation domain, A = adenylation domain, T = thiolation domain, TE = thioesterase domain, ACP = acyl carrier protein, TauD = hydroxylase, E = epimerase, AL = fatty acyl AMP-ligase

4. The post-assembly modification of siderophores

Following siderophore biosynthesis, the structure of a siderophore can be further modified both enzymatically and non-enzymatically resulting in a compound with altered physical properties. Commonly, these modifications result in a change in hydrophobicity of the siderophore without greatly altering its affinity for iron.

4.1 Pyoverdine maturation by PvdQ

Pyoverdine was discovered in 1892 and its involvement in bacterial iron uptake was established in the 1970s (Visca et al. 2007). Not until recently, however, was it known that pyoverdine is partially synthesized in the cytoplasm as an acylated precursor prior to formation of the chromophore (Hannauer et al. 2012; Yeterian et al. 2010). The acylated pyoverdine precursor is then transported into the periplasm where it encounters the acylase, PvdQ. PvdQ removes the fatty acid from the pyoverdine precursor, followed by chromophore formation by PvdO, PvdP and PvdN (**Figure 8**) (Yeterian et al. 2010). A knockout mutant of PvdQ did not affect the growth of *P. aeruginosa*, however, it did abolish pyoverdine production and release. The PvdQ knockout mutant also resulted in the impairment of swarming motility and biofilm formation in *P. aeruginosa* and an overall reduced virulence in a *Caenorhabditis elegans* infection model (Jimenez et al. 2010). As a result, finding small molecule inhibitors of PvdQ and altering its substrate specificity has been a topic of increasing interest to combat bacterial infection by pathogenic *Pseudomonas* species (Drake and Gulick 2011; Wurst et al. 2014; Clevenger et al. 2013; Koch et al. 2014).

Structural studies show PvdQ to be part of the Ntn-hydrolase superfamily of enzymes (Bokhove et al. 2010), which are autoproteolytically activated by removing a N-terminal signal sequence and a 23 residue spacer peptide to form a 18 kDa α-chain and a 60 kDa β-chain (Sio et al. 2006). Following self-activation, the serine nucleophile on the N-terminus of the β-chain is exposed. PvdQ was originally believed to be involved in quorum quenching due to the reactivity of PvdQ with acyl-homoserine lactones (Sio et al. 2006; Wahjudi et al. 2011; Huang et al. 2003). The location of *pvdQ* in the pyoverdine biosynthetic operon of *P. aeruginosa* PAO1, however, suggests PvdQ is involved with tailoring pyoverdine. The *pvdQ* gene is conserved among fluorescent *Pseudomonas* species although

pvdQ is not always contained in the pyoverdine biosynthetic operon. The *pvdQ* orthologues, however, are all iron-regulated and necessary for the production of pyoverdine (Koch et al. 2010).

PvdQ prefers median chain fatty acids *in vitro* (C12, C14), which corresponds to the fatty acid preference of module 1 from PvdL (Drake and Gulick 2011). It has been hypothesized that the acylated pyoverdine precursor prevents the siderophore from diffusing through the cytoplasm and periplasm. The acyl chain is thus proposed to hold the siderophore in the proper cellular location until biosynthesis is complete (Hannauer et al. 2012; Guillon et al. 2012).

4.2 Enzymatic modification of the marinobactins

In addition to *M. nanhaiticus* D15-8W, the marinobactin siderophores are also synthesized by *Marinobacter* sp. DS40M6 under low-iron conditions. During mid to late log phase bacterial growth, deacylated marinobactins (M_{HG}) are detected in the culture supernatant of both *Marinobacter* species. (Gauglitz et al. 2014). The PvdQ-like acylases, BntA, from *Marinobacter sp*. DS40M6 and MhtA from *M. nanhaiticus* D15-8W remove the fatty acid tail from the marinobactins to generate the marinobactin headgroup (Kem et al. 2015). MhtA does not, however, hydrolyze the Fe(III)-marinobactins suggesting a possible regulatory role in iron acquisition (Figure 10).

The fatty acid tail on the marinobactins allow for partitioning into membranes (Martinez and Butler 2007). In membrane-rich environments, removal of the fatty acid would release M_{HG} from membrane association to scavenge for iron without changing its iron binding properties. *Marinobacter* species are also known for their hydrocarbon degrading abilities and could possibly utilize the fatty acid products as a carbon source, as seen with other bacteria expressing Ntn-hydrolases (Huang et al. 2003; Lin et al. 2003).

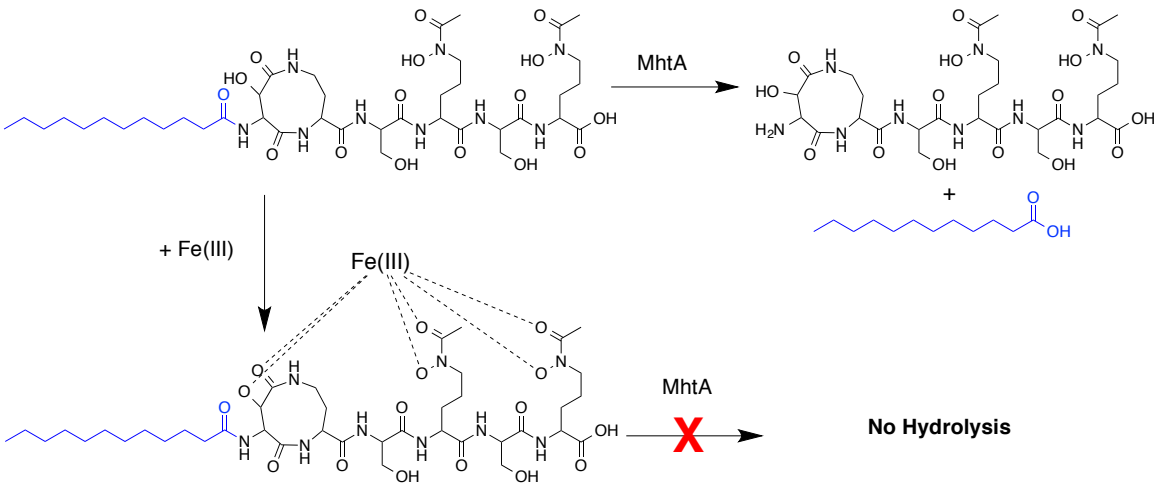

Figure 10. Hydrolysis of the marinobactins by MhtA. Reprinted with permission from Kem, M.P.; Naka, H.; Iinishi, A.; Haygood, M.G.; Butler, A. *Biochemistry* 2015, DOI: 10.1021/bi5013673. Copyright 2015 American Chemical Society.

4.3 Photochemistry of amphiphilic siderophores

Many marine siderophores possess α-hydroxycarboxylic acid functionalities to coordinate ferric iron. These α-hydroxycarboxylic acid moieties are part of β-hydroxyaspartic acid or citric acid, and are photoreactive when coordinated to Fe(III) (Barbeau et al. 2001). Photolysis induces ligand oxidation and the release of CO_2 in conjunction with the reduction of Fe(III) to Fe(II) (Kupper et al. 2006; Martin et al. 2006; Ito and Butler 2005). During photolysis, the citrate ligand is converted to 3-ketoglutaric acid, which can undergo a keto-enol tautomerization to the enolate form. The enolate form is more prevalent in aqueous solutions and can chelate Fe(III) (Butler and Theisen 2010; Kupper et al. 2006). The photolysis of aerobactin has been studied most extensively and can be monitored by the disappearance of the α-hydroxycarboxylic acid-to-Fe(III) charge transfer band around 300 nm. UV-photolysis studies of the Fe(III)-ochrobactins and Fe(III)-snychobactins, which are acylated analogues of aerobactin, produced similar results..

Many structurally characterized amphiphilic siderophores also contain β-hydroxyaspartic acid residues, including the marinobactins, aquachelins, loihichelins, cupriachelin, taiwachelin and serobactins (Martinez et al. 2000; Homann et al. 2009; Kreutzer et al. 2012; Kreutzer and Nett 2012; Rosconi et al. 2013). The aquachelins produced by *Halomonas aquamarina* strain DS40M3 have a N-terminal β-hydroxyaspartic acid residue connected to the fatty acid. Photolysis of the ferric-aquachelins results in oxidative cleavage of the ligand, forming a hydrophilic peptide fragment without the fatty acid (**Figure 11**A). The hydrophilic peptide fragment is still able to chelate

Fe(III), however, with a somewhat lower conditional stability constant ($K_{FeL,Fe}{}'^{cond}$ = $10^{11.5}$ M^{-1} vs $10^{12.2}$ for the unmodified siderophore) (Barbeau et al. 2003). This decrease in the stability constant corresponds to the loss of the β-hydroxyaspartic acid residue, which is one of the Fe(III) chelating groups.

Cupriachelin has two β-hydroxyaspartic acid residues in its backbone; however, photo induced cleavage was only seen with the central β-hydroxyaspartic acid in the peptide backbone (**Figure 11**B) (Kreutzer et al. 2012). Photo induced hydrolysis of the cupriachelin backbone results in the formation of Fe(II), a fatty acid containing peptide, and a small hydrophilic peptide.

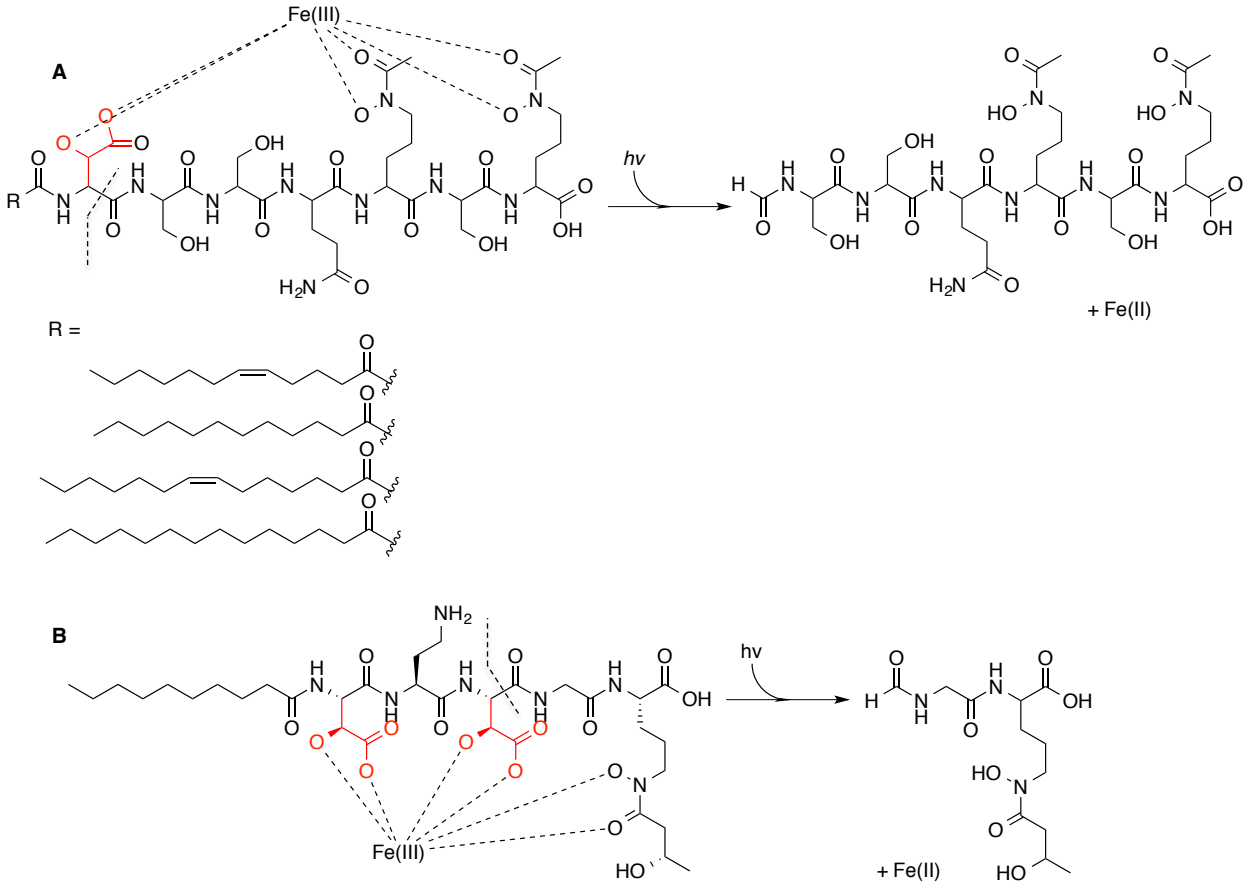

Figure 11. Photolysis of Fe(III) complexes to β-OH-Asp-containing siderophores (A) Photolysis of the aquachelins results in the loss of the fatty acid moiety and production of a hydrophilic headgroup, which is still able to chelate Fe(III). (B) Cupriachelin has two β-hydroxyaspartic acid residues, however, only the central β-hydroxyaspartic acid residue results in peptide cleavage.

5. Conclusion and perspectives

The wealth of microbial genomic information has clearly facilitated the discovery of new amphiphilic siderophores, not only in marine bacteria, but also in terrestrial microbes. Often fatty acid activation is required to

initiate biosynthesis of the acyl-peptidic siderophores, through one of several different mechanisms. Fatty acyl-AMP ligases activate a fatty acid and initiate the biosyntheses of pyoverdine (Figure 8), marinobactins, taiwachelins and serobactins (Figure 9C-E). External ligases that reside outside the biosynthetic gene clusters for cupriachelins (Figure 9A), amphibactins (Figure 9B) and amphi-enterobactins (Figure 6) are thought to initiate siderophore biosynthesis through initial fatty acid activation. The biosynthesis of the mycobactins is unique among the acyl siderophores described here in that acylation does not initiate biosynthesis of the siderophore, but rather is incorporated during peptide chain elongation through recognition of the L-N^6-OH-N^6-acyl-Lys unit by the A domain of MbtE (Figure 7).

These acyl peptidic siderophores may be further modified enzymatically through fatty acid hydrolysis to produce a more hydrophilic compound which retains its ferric chelating abilities as occurs with the marinobactins (Figure 10) and pyoverdines (Figure 8). Acyl siderophore hydrophobicity can also be altered photochemically in the Fe(III) complexes of β-hydroxyaspartic acid-containing siderophores of the aquachelins and cupriachelin (Figure 11) through oxidative loss of the N-terminal β-hydroxyaspartic acid, as well as N-appended fatty acid. The ferric complexes of the taiwachelins, serobactins, and marinobactins are also photoreactive.

Clearly the amphiphilic nature of acylated siderophores is advantageous in the case of the mycobactins where amphiphilicity provides the siderophore with the ability to diffuse freely between membranes and media allowing the siderophore to acquire iron from macrophages. In the case of the acyl pyoverdine precursor, the fatty acid is proposed to anchor the growing chain in a membrane during biosynthesis, to prevent its diffusion. Fatty acid hydrolysis of the marinobactins during late log phase of growth by an Ntn-hydrolase, which also hydrolyses acyl-homoserine lactones may provide a signaling function. Yet for the majority of the other siderophores described herein the biological function of siderophore acylation remains to be revealed.

Bioinformatics is clearly useful in identifying biosynthetic gene clusters for acyl peptidic siderophores, yet many question remain surrounding what controls the nature of the suite of acylated peptides. While the amphibactins are produced as a very large suite with fatty acid variation from C12 to C18 and with saturated, hydroxylated or desaturated fatty acids, other acyl peptidic siderophores such as cupriachelin or the serobactins are produced as a single acylated siderophore or as a small suite with only a couple of members. We look to future investigations to uncover the factors and the mechanisms governing the range of fatty acid incorporation into acyl peptidic siderophores.

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