Biosynthesis of Amphi-enterobactin Siderophores by *Vibrio harveyi* BAA-1116: Identification of a Bifunctional NRPS Condensation Domain

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Supporting Information Placeholder

ABSTRACT: The genome of *Vibrio harveyi* BAA-1116 contains a nonribosomal peptide synthetase (NRPS) gene cluster (*aebA-F*) resembling that for enterobactin, yet enterobactin is not produced. A gene predicted to encode a long chain fatty acid CoA ligase (FACL), similar to enzymes involved in the biosynthesis of acyl peptides, resides 15 kb away from the putative enterobactin-like biosynthetic gene cluster (*aebG*). The proximity of this FACL gene to the enterobactin-like synthetase suggested that *V. harveyi* may produce amphiphilic enterobactin-like siderophores. Extraction of the bacterial cell pellet of *V. harveyi* led to the isolation and structure determination of a suite of eight amphi-enterobactin siderophores comprised of the cyclic lactone of *tris*-2,3-dihydroxybenzoyl-L-serine and acyl-L-serine. The FACL knockout mutant, Δ*aebG V. harveyi*, and the NRPS knockout mutant, Δ*aebF V. harveyi*, do not produce amphi-enterobactins. The amphi-enterobactin biosynthetic machinery was heterologously expressed in *E. coli* and reconstituted *in vitro*, demonstrating the condensation domain of AebF has unique activity, catalyzing two distinct condensation reactions.

Vibrio harveyi BAA-1116 (recently reclassified as *V. campbellii*[1,2]) is widely used to study quorum sensing, due to its quorum-regulated bioluminescence.[3,4] Quorum sensing in *V. harveyi* is known to regulate siderophore production, but the identity of the siderophore had not been determined.[3] Siderophores are low molecular weight iron(III) chelators, that solubilize and transport iron(III) into the cell.[5] Siderophores contribute to competitiveness in environmental bacteria, and serve as virulence factors in pathogens, including *Vibrios*.[6] Microbial colonization of an animal host depends critically on the ability to obtain sufficient iron, yet is also challenged by the low levels of available iron in the host environment.[7]

The genome of *V. harveyi* strain ATCC BAA-1116 contains a nonribosomal peptide synthetase (NRPS) gene cluster resembling that for enterobactin biosynthesis (Figure 1, Table S1), yet we did not detect enterobactin in cultures of *V. harveyi*. Enterobactin, the lactone of *tris*-2,3-dihydroxybenzoyl-L-serine, is the prototypic catecholate siderophore produced by many bacteria, most notably *Escherichia coli*[8] (Figure 1C). An encoded long chain fatty acid CoA ligase (FACL) is present in the *V. harveyi* genome near the putative enterobactin biosynthetic gene cluster (Figure 1B). This enzyme is predicted to be similar to enzymes that are involved in the acylation of peptides, often located in the vicinity of the NRPS cluster.[9,10,11]

The organization of the *V. harveyi* gene cluster, specifically the FACL nearby the NRPS genes, in conjunction with the continued discovery of new N-terminally acylated peptide siderophores,[11,12] led us to hypothesize that *V. harveyi* produces acylated enterobactin-like siderophores. We report herein the structural characterization of the amphi-enterobactin siderophores produced by *V. harveyi* ATCC BAA-1116, in addition to the biosynthetic genes, designated as the *aeb* gene cluster (Figure 1B, Table S1). Through heterologous expression and *in vitro* reconstitution of amphi-enterobactin biosynthesis, we reveal unique dual activity of the single condensation domain of the NRPS protein, AebF.

V. harveyi ATCC BAA-1116 was grown in a low iron synthetic seawater medium. After two days, the culture was harvested by centrifugation, the bacterial cell pellet was extracted with methanol and the extract was purified with solid phase adsorption and RP-HPLC (λ 310 nm for catechol absorption). Eight peaks gave a positive CAS assay response for iron binding, consistent with the presence of apo-siderophores (Figure 2).[13] ESI-MS of these compounds showed a range in mass from *m/z* 927 – *m/z* 987 (Table S2, Figures S1-S8). Enterobactin (*m/z* 670 $[M+H]^+$) was not detected in either the culture supernatant or the bacterial cell pellet.

ESI-MS/MS of the eight compounds produced a similar pattern, with three major daughter ions at *m/z* 670, *m/z* 447 and *m/z* 224 ($[M+H]^+$) (Figure S4A), as observed in the ESI-MS/MS of enterobactin.[14] However, in each spectrum, additional fragment ions were observed, varying in mass amongst the compounds, analogous in pattern to other siderophore suites that are N-terminally acylated with differing fatty acids[15,16] (Figure S4A). The ESI-MS/MS pattern is consistent with the predicted amphi-enterobactin structure, with fragmentation along the lactone backbone producing ions containing both, L-Ser-DHBA and L-Ser-fatty acid. The attached fatty acid varied

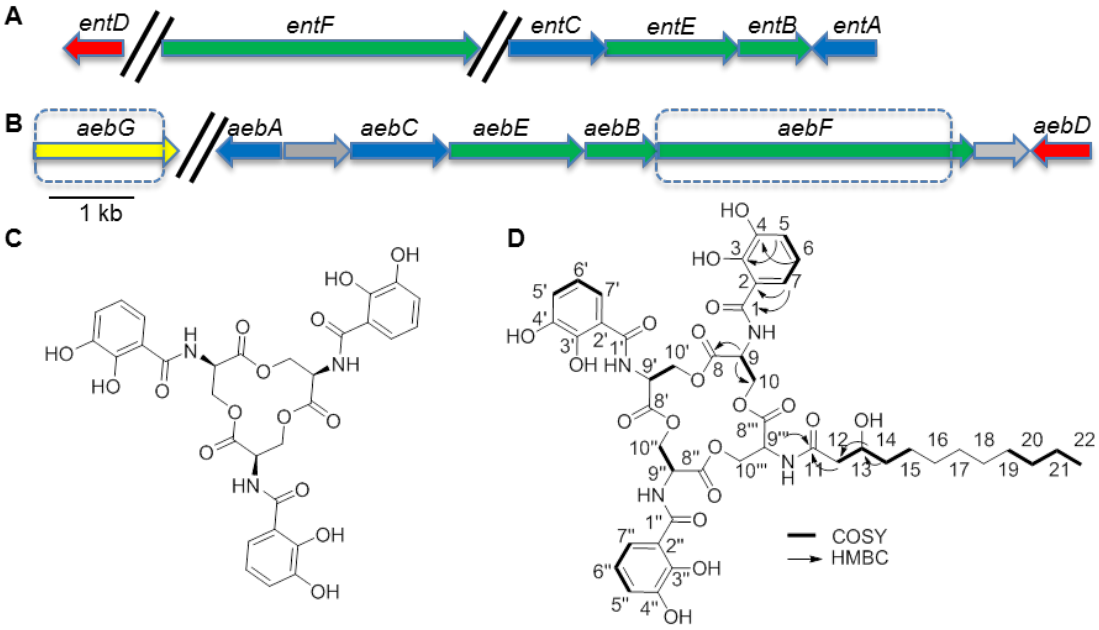

Figure 1. **A.** Schematic of the genes composing the enterobactin biosynthetic cluster in *E.coli*. **B.** Schematic of the genes composing the putative amphi-enterobactin cluster in *V. harveyi*: yellow, FACL; blue, DHBA biosynthetic proteins: green, NRPS proteins, red, P-pant transferase (see Table S1 for details). Boxes with dashed lines indicate the genetic regions deleted in this study. **C.** Enterobactin. **D.** Amphi-enterobactin C12-OH (**1**; peak 4 in Figure 2) depicted with ^{1}H-^{1}H correlations (bold lines) and key HMBC (H→C) correlations.

among the amphi-enterobactins with chain length (C10-C14), degree of unsaturation and whether the fatty acid was hydroxylated (Table S2).

The structure of amphi-enterobactin C12-OH (**1**; peak 4 in Figure 2) was further elucidated with high-resolution electrospray ionization mass spectrometry (HIRESIMS) and ^{1}H, ^{13}C and 2D (COSY, TOCSY, HMBC) NMR (Figure 1D). HIRESIMS established the mass of the molecular ion $[M+Na]^{+}$ of **1** as 977.3272, corresponding to a molecular formula of $C_{45}H_{54}N_4O_{19}Na$ (Figure S9). NMR of **1** revealed the ^{1}H resonances to be similar to reported chemical shifts of enterobactin,[17] with the exception of the aliphatic peaks in the ^{1}H spectrum of **1**, resulting from the presence of the fatty acid. Integration from 0.89 ppm to 1.5 ppm corresponded to the 19 aliphatic protons on C14-C22. The proton of the hydroxy-bearing carbon at C13 generated a multiplet at 3.91 ppm, and the diastereotopic protons of C12 were split into a doublet of multiplets at 2.27 ppm and 2.34 ppm, authenticating the presence of 3-hydroxydodecanoic acid (Figure S10). HMBC correlations showed the connectivity of both, the α-^{1}H serine and the diastereotopic C12 protons, to the carbonyl carbon of 3-hydroxydodecanoic acid, confirming the structure of **1**. A more subtle difference in the NMR spectra of **1** is evident in the distinct sets of chemical shifts observed for the three DHBA units and four Ser residues, arising from the asymmetry of **1**, compared to the symmetry of enterobactin, which shows a single set of resonances for the DHBA-Ser unit (Table S3, Figures S10-S13).

The proposed amphi-enterobactin biosynthetic pathway contains six genes (*aebA-F*) predicted to encode proteins that are homologous to the well-characterized enterobactin biosynthetic machinery (Figure 1, Table S1). In *E. coli*, EntC, EntB (N-terminal domain) and EntA convert chorismate to 2,3-dihydroxybenzoic acid (DHBA); EntE, EntB (C-terminal domain), EntD and EntF, four nonribosomal peptide synthetase (NRPS) proteins, catalyze formation of the DHBA-L-Ser unit, polymerize three of these units and cyclize the trimer to form the lactone backbone.[8] However, a distinguishing feature in *V. harveyi* is the presence of the proximal *aebG* gene, predicted to encode a FACL. FACL enzymes have been demonstrated to activate fatty acids to fatty acyl-CoA thioesters prior to incorporation into acylated nonribosomal peptides.[9,10] Therefore, we predict that the *aebA-F* gene cluster is responsible for the formation of the amphi-enterobactin core structure and the *aebG* gene is engaged in the acylation of the serine residue.

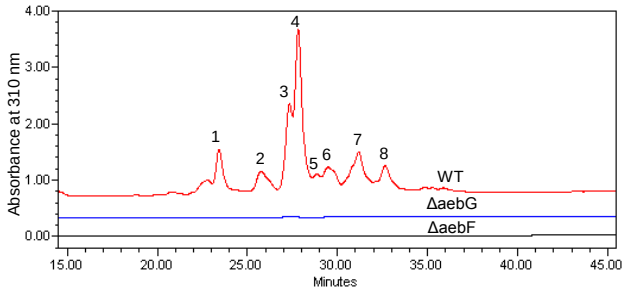

Figure 2. RP-HPLC chromatogram of amphi-enterobactin extraction samples: wt BAA-1116 strain (red), Δ*aebG* (blue), Δ*aebF* (black). Flow rate= 7 ml/min, solvent A=H_2O, solvent B= MeOH.

To investigate the postulated amphi-enterobactin biosynthetic pathway, knockout mutants of the putative FACL (*aebG*) and NRPS (*aebF*) genes were constructed in *V. harveyi* BAA-1116 and the knockout strains were grown under iron-limiting conditions to promote amphi-enterobactin production. RP-HPLC analysis of the Δ*aebG V. harveyi* and Δ*aebF V. harveyi* cell pellet and supernatant extracts reveals the abolition of the amphi-enterobactins (Figure 2). Moreover, enterobactin was not detected. *AebG* gene complementation restored amphi- enterobactin production (Figure S15). The complementation confirms that this gene is necessary for the

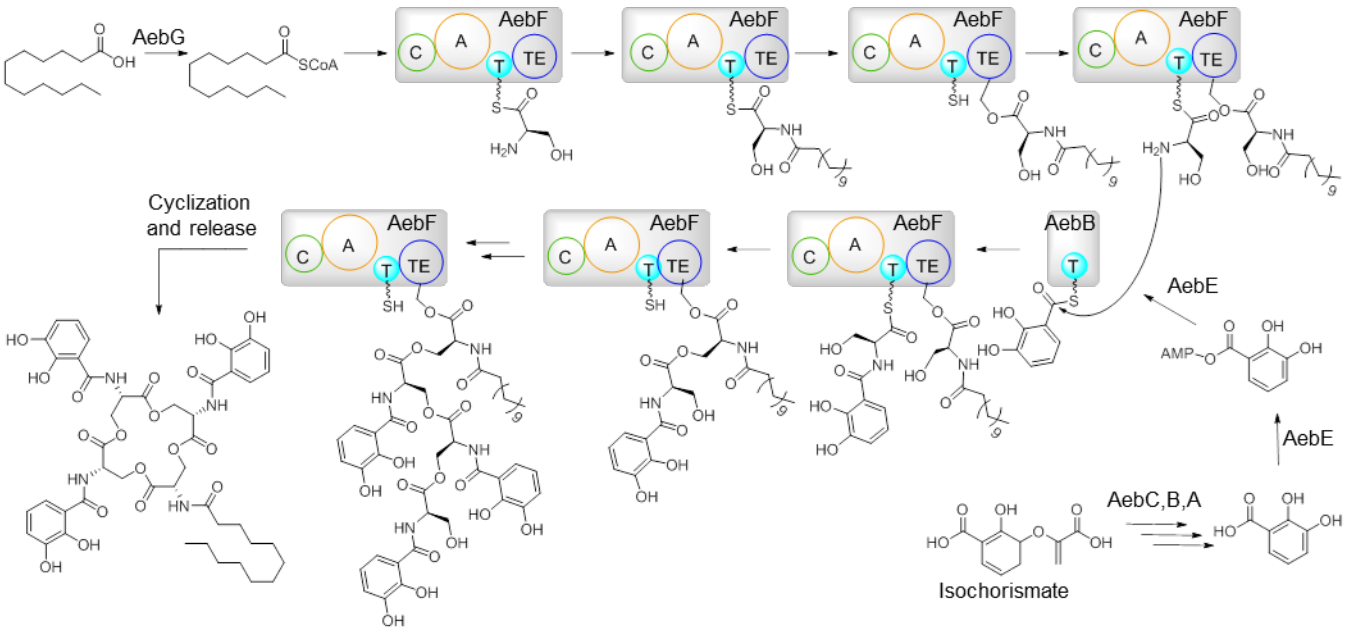

Figure 3. Proposed biosynthesis of the amphi-enterobactin siderophores based on the iterative enterobactin "waiting room" model[18]: C, condensation domain; A, adenylation domain; T, thiolation domain; TE, thioesterase domain.

production of the acylated enterobactin siderophores. We propose that AebG is required to activate the fatty acid as a fatty acid CoA thioester prior to incorporation[9,10] and that AebF catalyzes amide bond formation and cyclization of the lactone backbone.

Bioinformatic analysis of AebF, including sequence alignment and structure prediction, indicates a linear four-domain NRPS organization: C-A-T-TE; C, condensation; A, adenylation; T, thiolation; TE, thioesterase, similar to the enterobactin NRPS, EntF (54% similarity) (Table S1). Additionally, the A domain of AebF contains the identical specificity-conferring residues as EntF (DVWHFSLV), allowing prediction of L-Ser specific activation.[19,20] In the biosynthesis of enterobactin, the one C domain of EntF catalyzes amide bond formation between DHBA tethered to the 4'-phosphopantetheine (DHBA-*S*-P-pant-EntB) arm of the aryl carrier protein, EntB and L-Ser-*S*-P-pant-EntF. However, in the amphi-enterobactins, two types of amide bonds are present, L-Ser-DHBA, and L-Ser-fatty acid, while domain prediction analysis shows only one C domain. To probe the activity of the ~148 kDa AebF protein *in vitro*, the *aebF* gene was cloned and recombinantly expressed in *E. coli* with a C-terminal His-tag for purification (Figure S16). L-Ser activation of AebF was tested using the nonradioactive malachite green colorimetric assay,[21,22] confirming the predicted specificity. To evaluate the N-acylation activity of the C domain, *holo*-AebF was generated *in situ* via treatment with coenzyme A and recombinant P-pant transferase, Sfp, from *B. subtilis*.[23] Condensation between lauroyl CoA and L-serine was tested by incubating them with *holo*-AebF in the presence of Mg^{2+} and ATP for 2 hours at room temperature. After hydrolysis of the acyl-serine from AebF with KOH, the product was analyzed with LC-MS and LC-MS/MS. The $[M+H]^+$ lauroyl-Ser ion was detected at *m/z* 288.2 (Figure S17). The fragmentation pattern of the tandem spectrum is consistent with lauroyl-Ser (Figure S17). The control reaction without AebF produced no acylated product, demonstrating that the sole NRPS C domain catalyzes the fatty acid acylation of L-serine using fatty acyl-CoA thioester as a donor.

With AebF shown to be capable of producing the acylated serine residue, DHBA-Ser amidation by the C domain of AebF was examined. Based on the homology of AebE and AebB to the well-characterized components of the enterobactin synthetase, EntE and EntB (64% and 66% similarity, respectively), AebE and AebB were predicted to be necessary for DHBA activation: AebE functioning as the DHBA-AMP ligase and AebB as the DHBA carrier protein. Once DHBA is loaded on the P-pant arm of AebB, we hypothesized that the C domain of AebF would catalyze DHBA amide formation with L-Ser-*S*-P-pant-AebF, homologous to the C domain of the EntF enterobactin synthetase.[24] The *aebE* and *aebB* genes were cloned and recombinantly expressed for *in vitro* studies. AebB was obtained as a C-terminal His-tagged fusion protein and AebE as a N-terminal thioredoxin-His tagged construct (Figures S18 and S19). DHBA specific activation of AebE was established through the nonradioactive malachite green colorimetric assay.[21,22] To determine the DHBA-Ser amidation activity of AebF, *holo*-AebB, *holo*-AebF, and AebE were incubated with L-Ser, DHBA, Mg^{2+} and ATP at room temperature. However, following work-up, no DHBA-Ser product was detected. To determine if the fatty acid CoA thioester is necessary to initiate the biosynthesis of the amphi-enterobactin siderophores, lauroyl CoA was added to the reaction mixture above and extracted with ethyl acetate after incubation. The $[M+H]^+$ amphi-enterobactin C12 ion was detected at *m/z* 939.3 via LC-MS and LC-MS/MS, identifiable by the fragmentation pattern of the tandem mass spectrum (Figure S20). Control reactions without ATP did not produce detectable amounts of siderophore.

In sum we have isolated and characterized a suite of unprecedented amphi-enterobactin siderophores, originally predicted from the bioinformatic analysis of the *V. harveyi* ATCC BAA-1116 genome. Eight amphi-enterobactins were identified with various fatty acid appendages ranging in length (C10-C14), degree of unsaturation and hydroxylation, dominated by amphi-enterobactin C12-OH, **1** (Figure 1D). Amphi-enterobactin production is induced under low iron conditions, suggesting they are important for iron acquisition in *V. harveyi* and we propose that amphi-enterobactin produc-

tion is conserved among many marine *Vibro* spp. based on genome mining. An emerging characteristic of marine bacterial siderophores is the prevalence of acyl siderophores comprised of a peptidic iron-binding head group appended by one of a series of long chain fatty acids of varying degrees of saturation and hydroxylation.[5,15,16] Acyl siderophore precursors have also recently been discovered in the biosynthesis of pyoverdine from the human pathogen *Pseudomonas aeruginosa*.[25,26] The fatty acid group is proposed to anchor the acyl pyoverdine precursor in the bacterial membrane, where the biosynthesis is suggested to occur.[26,27] Enterobactin biosynthetic proteins have also been proposed to be membrane associated.[28] While amphi-enterobactin biosynthetic proteins are predicted to be cytoplasmic, the cellular location has not been experimentally determined, and a membrane-associated biosynthetic strategy could take place in *V. harveyi* similar to that in *P. aeruginosa*.

The amphi-enterobactin biosynthetic enzymes are similar to the enterobactin biosynthetic enzymes, however we have shown that the activity of the C domain on the NRPS protein, AebF, differs. In the enterobactin synthetase, EntF, the C domain catalyzes amide bond formation between DHBA-*S*-P-pant-EntB and L-Ser-*S*-P-pant-EntF. Our analysis shows that the C domain of the analogous amphi-enterobactin biosynthetic protein, AebF, accepts both DHBA-*S*-P-pant-AebB and fatty acyl-CoA thioesters as donors in the DHBA amidation and fatty acid acylation of L-Ser-*S*-P-pant-AebF, demonstrating novel bifunctional activity. We propose that the fatty acyl-CoA thioester acylation of L-Ser-*S*-P-pant-AebF initiates biosynthesis, based on the absence of DHBA-Ser product formation when the fatty acid CoA thioester is omitted. We also propose that AebG, a putative FACL located near the cluster, provides the acyl-CoA thioester substrate. AebG is necessary for biosynthesis, as demonstrated by the absence of the amphi-enterobactin siderophores, as well as any DHBA-Ser fragments, in the Δ*aebG V. harveyi* BAA-1116 cell pellet extract or supernatant. The reactions catalyzed by AebF provides the first biochemical demonstration of a C domain that performs condensation reactions using two very different donor substrates, although similar activity is predicted in the initial C domain in the biosynthetic pathway of the acylated peptide ramoplanin.[29] Many acylated peptides have antimicrobial properties that are highly dependent upon the nature of the fatty acid appendage.[30] Continued investigations into these promiscuous starter C domains will provide a platform for bioengineering new acylated peptides.

Based on the results of this study, we propose a putative amphi-enterobactin biosynthetic pathway constructed off the enterobactin "waiting room" model of biosynthesis[18] (Figure 3). In this model the TE domain serves as a "waiting room" for the L-Ser-DHBA during each iterative round. For the biosynthesis of the amphi-enterobactins, the TE domain of AebF must be able to harbor the 16-member tetralactone backbone of the amphi-enterobactins, versus the 12-member trilactone of enterobactin, suggesting an increased degree of elasticity for the TE domain of AebF. Future crystallographic data will allow for the structural comparison of TE domains and may provide insight into the unique characteristics of AebF.

ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization data for **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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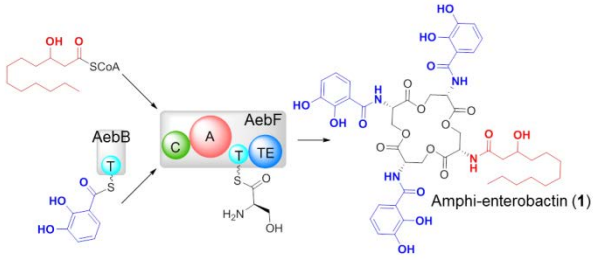
SCoA
AebB
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Amphi-enterobactin (1)