Catalytic Mapping of the Vibriobactin Biosynthetic Enzyme VibF†

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Received September 26, 2001; Revised Manuscript Received October 26, 2001

ABSTRACT: The iron-chelating catechol siderophore vibriobactin of the pathogenic *Vibrio cholerae* is assembled by a four-subunit, ten-domain nonribosomal peptide synthetase system, VibE, VibB, VibH, and VibF, using 2,3-dihydroxybenzoate and L-threonine as precursors to two (dihydroxyphenyl)-methyloxazolinyl groups in amide linkage on a norspermidine scaffold. We have utilized site-specific and domain-deletion mutagenesis to map the heterocyclization and primary and secondary amine acylation activities of the six-domain (Cy1-Cy2-A-C1-PCP-C2) VibF subunit. We have found that Cy2 is capable of and limited to the condensation (amide bond formation) step of the three-step heterocyclization process, while Cy1 is capable of and limited to the final processing (cyclization/dehydration) steps to the completed heterocycle. Additionally, we have observed that the C2 domain functions in both N_9 (primary amine) acylation and N_5 (secondary amine) acylation of the (dihydroxybenzoyl)norspermidine substrate, leaving no catalytic role for the C1 domain, a conclusion confirmed with the formation of vibriobactin in a C1-deficient system. Thus VibF is an NRPS with two domains, Cy1 and Cy2, that perform a function otherwise performed by one and with one domain, C2, that performs a function otherwise performed by two. While C2 appeared to tolerate uncyclized threonine in place of the usual heterocycle in primary amine acylation, it refused this replacement in the corresponding donor substrate in secondary amine acylation.

Vibriobactin (**1**) is a low molecular weight, iron-chelating catechol siderophore produced by *Vibrio cholerae* in conditions of iron limitation (e.g., during infections in vertebrates) where it contributes to a system of iron acquisition essential to virulence (*1*, *2*). It is the assembled product of the four-component NRPS[1] system VibE, VibB, VibH, and VibF and is composed of three molecules of 2,3-dihydroxybenzoate (DHB), two molecules of L-threonine (L-Thr), and the symmetric triamine norspermidine (NS). We have elucidated the biosynthetic route to vibriobactin from these starting molecules (*3*, *4*) and have demonstrated the critical function of the six-domain VibF component (Cy1-Cy1-A-C1-PCP-C2, Figure 1) in L-threonine activation by the adenylation (A) domain, self-acylation of the peptidyl carrier protein (PCP) domain, capture of activated DHB and subsequent heterocyclization to yield the (dihydroxyphenyl)methyloxazolinyl-*S*-VibF (DHP-mOx-*S*-VibF) species by the heterocyclization (Cy) domains, and transfer of this acyl species twice to N_9 and N_5 of DHB-N^1-norspermidine (DHB-NS, **3**) by the condensation (C) domains to give **2** and vibriobactin, respectively. DHB-NS itself is manufactured independently

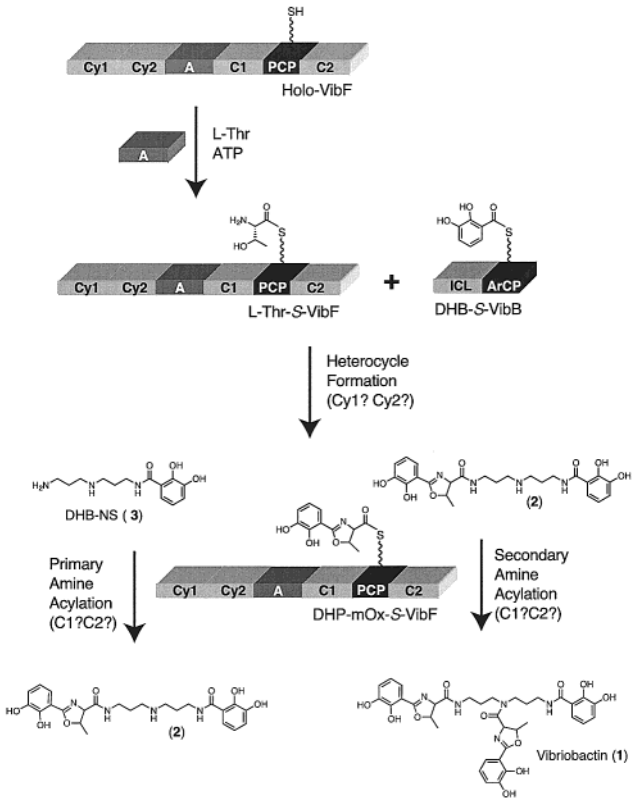

FIGURE 1: Biosynthesis of vibriobactin from L-Thr, ATP, DHB-*S*-VibB, and DHB-NS by VibF. DHB-NS is acylated at both N_9 (primary) and N_5 (secondary) amines by activated DHP-mOx generated by DHB-*S*-VibB, L-Thr-*S*-VibF, and VibF heterocyclization activity.

† This work has been supported by the National Institutes of Health (Grant AI042738 to C.T.W.). C.G.M. is a Fellow of the National Science and Engineering Research Council of Canada. N.J.H. is funded by a National Defense Science and Engineering (NDSEG) Fellowship.

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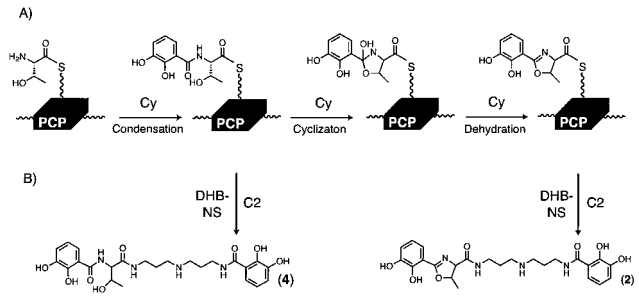
[1] Abbreviations: A, adenylation domain; ArCP, aryl carrier protein; C, condensation domain; CoA, coenzyme A; Cy, heterocyclization domain; DHB, 2,3-dihydroxybenzoate; DHB-NS, N^1-(2,3-dihydroxybenzoyl)norspermidine; DHP, 2,3-dihydroxyphenyl; DTT, dithiothreitol; ICL, isochorismate lyase; IPTG, isopropyl β-D-thiogalactopyranoside; mOx, 5-methyloxazoline; NRPS, nonribosomal peptide synthetase; PCP, peptidyl carrier protein; PP_i, inorganic pyrophosphate; PPTase, phosphopantetheinyl transferase; TE, thioesterase domain; TCEP, tris-(carboxyethyl)phosphine.

10.1021/bi011852u CCC: $22.00 © 2002 American Chemical Society
Published on Web 11/27/2001

Scheme 1



from DHB-*S*-VibB and norspermidine by the stand-alone condensation domain VibH (*3*).

Although we have examined the acceptor substrate specificity of the heterocyclization domains (*5*) and have established that the C2 domain of VibF is competent for its N_9 primary amine acylation activity, assignment of heterocyclization and N_5 secondary amine acylation activities to their corresponding domains has yet to be accomplished.

Heterocyclization domains are found in NRPS systems that synthesize a variety of molecules, including the thiazoline- and thiazolidine-containing siderophore yersiniabactin (*6*), the thiazoline-containing antitumor epothilone and antibiotic bacitracin (*7*, *8*), and the oxazoline-containing siderophore mycobactin (*9*). In all of these NRPS systems, a single heterocyclization domain is responsible for the three required chemical steps: condensation of the β-nucleophile-containing amino acid (the acceptor) with the activated upstream acyl group (the donor), cyclization via attack across the newly formed amide bond, and dehydration to form the stabilized dihydro heterocycle found in the final product (Scheme 1A). Unlike these NRPS systems, however, VibF is unusual in that it contains a tandem pair of heterocyclization domains, suggesting a redundancy in activity or a nonfunctional domain, a feature common in PKS systems (*10*, *11*). Also unusual is the positioning of the C1 domain in the linear domain order of VibF, deviating from the C(y)-A-PCP paradigm of NRPS systems. The role of C1 has not been established nor has the domain assignment in the N_5 secondary amine acylation activity that produces vibriobactin. In this study, we have utilized site-directed mutagenesis of core amino acids, as well as entire domain deletion mutants, to map catalytic activities of VibF to its bioinformatically assigned domains. We have discovered that both Cy domains are required for full heterocyclization activity, while only one C domain is capable of both N_9 primary and N_5 secondary amine acylation activities.

EXPERIMENTAL PROCEDURES

Materials and General Methods. 2,3-DHB, coenzyme A, and all amino acids were purchased from Sigma-Aldrich Chemical Co. ATP was purchased from Boehringer Mannheim. TCEP was purchased from Fluka. DHP-mOx-NS-DHB was obtained as described previously (*4*). Standard recombinant DNA techniques and microbiological procedures were performed as described (*12*). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Plasmid vectors were purchased from Novagen. Pfu polymerase and competent *Escherichia coli* was purchased from Stratagene. Oligonucleotide primers were purchased from Integrated DNA Technologies, and DNA sequencing to verify the fidelity of amplification was performed on double-stranded DNA by the Molecular Biology Core Facilities of the Dana Farber Cancer Institute (Boston, MA). Ni-NTA Superflow resin was purchased from Qiagen.

Table 1: DNA Primers Used in This Study

primer name	nucleotide sequence[a]
D133A5′f	5′-CTTTAAGAAGGAGATATACATATG-3′
D133A5′r	5′-ACACGCAATCAT**AGC**CGCATCAATGTC-3′
D133A3′f	5′-CACATTGATGCG**GCT**ATGATTGCGTGT-3′
D133A3′r	5′-GGGAGACGCAGCATCAATAGGTAC-3′
D138A5′r	5′-GAAAACTTTGTGC**AGC**ACACGCAATCATATC-3′
D138A3′f	5′-GATATGATTGCGTGT**GCT**GCACAAAGTTTTC-3′
ΔCy1−5′	5′-GATATACATATGCAGCAAAGCGTACAA-3′
D590A5′r	5′-GACAATCAAAGC**AGC**AAAGCTGGTCAA-3′
D590A3′f	5′-TTGACCAGCTTT**GCT**GCTTTGATTGTC-3′
D595A5′r	5′-ATGGGTGCGACC**AGC**GACAATCAAAGC-3′
D595A3′f	5′-GCTTTGATTGTC**GCT**GGTCGCACCCAT-3′
E1552A5′f	5′-TCCCGTATGGTCGAC-3′
E1552A5′r	5′-ATCCCATTG**CGC**GCTGATAAT-3′
E1552A3′f	5′-ATTATCAGC**GCG**CAATGGGAT-3′
E1552A3′r	5′-TGAAGGATCCACTAACCA-3′
H2084A5′f	5′-ACTTGGTTAGTGGATCCT-3′
H2084A5′r	5′-CAACACAAT**AGC**GTGGAACAG-3′
H2084A3′f	5′-CTGTTCCAC**GCT**ATTGTGTTG-3′
H2084A3′r	5′-GTTTAGCAGCCTAGGTAT-3′

[a] Ala codon is in bold; restriction sites are underlined.

HPLC Sample Preparation and Analysis. Reactions to be analyzed by HPLC were quenched with 9 volumes of ice-cold methanol, and precipitant was removed by centrifugation at 11600*g* for 30 min at 4 °C. Samples were dried under vacuum at 32 °C and suspended in 150 μL of 15% acetonitrile for injection onto a C18 Vydac small-pore column on a Beckman System Gold. Peaks were eluted at 1 mL/min in a gradient from 10% to 90% acetonitrile in 0.08% TFA over 23 min. Product elution was monitored at 254 nm. Peak integration values were converted to nanomoles of product on the basis of standard curves generated with DHB-octylamine and DHP-mOx-NS-DHB as described previously (*4*).

Bioinformatic Analysis. Amino acid alignments were performed with DNAStar MegAlign software using the Clustal X algorithm with a gap penalty of 10 and a gap length penalty of 10.

Construction of VibF Mutant Plasmids. Plasmids containing VibF point-mutant genes were created by SOE mutagenesis (*13*) of the wild-type plasmid pVibF (*4*) using the primers listed in Table 1 and the primer pairing in Table 2. The deletion mutant ΔCy1 was created from the primer pairing given in Table 2. Amplification products were digested with the appropriate restriction endonucleases and ligated to the complementing product of similarly digested pVibF. Ligation products were transformed into *E. coli* BL21(DE3) for heterologous expression and their identities confirmed by DNA sequencing. The double mutant pVibFD133A/D590A was created by transferring a *Bgl*I fragment from pVibFD133A to pVibFD590A, while the double mutant pVibFE1552A/H2084A was created by transferring an *Eco*RV/*Bam*HI fragment from pVibFE1552A to pVibFH2084A. The host vector for all of these clones was pET37 in which a C-terminal $(His)_8$ tag is appended to the expression product.

Purification of VibF, CyCyA, and PCPC2. VibF wild-type and mutant enzymes were purified from *E. coli* BL21(DE3)

Table 2: PCR Amplification Primer Pairs in the Construction of VibF Mutants

mutant name	primers used
D133A	D133A5′f and D133A5′r
	D133A3′f and D133A3′r
D138A	D133A5′f and D138A5′r
	D138A3′f and D133A3′r
ΔCy1	ΔCy15′ and D133A3′r
D590A	D133A5′f and D590A5′r
	D590A3′f and D133A3′r
D595A	D133A5′f and D595A5′r
	D595A3′f and D133A3′r
E1552A	E1552A5′f and E1552A5′r
	E1552A3′f and E1552A3′r
H2084A	H2084A5′f and H2084A5′r
	H2084A3′f and H2084A3′r

containing the appropriate plasmid. Culture (3 × 1 L) in Luria−Bertani broth supplemented with kanamycin (50 μg/mL) and 5 mM $MgCl_2$ was grown for 48 h at 250 rpm, 22 °C, with expression from the T7 promoter occurring in the absence of added IPTG. Harvested cells were lysed by a French press into 20 mM Tris, pH 8.0, 500 mM NaCl, and 2 mM $MgCl_2$, the lysate was clarified by ultracentrifugation, and the supernatant was incubated with 0.25 mL of Qiagen Ni-NTA Superflow resin for 5 h. VibF was eluted with a step gradient of 10 column volumes of lysis buffer containing 25−200 mM imidazole in 25 mM increments, and the eluant was analyzed by 9% SDS−PAGE. Fractions containing purified VibF were pooled and dialyzed against 20 mM Tris, pH 8.0, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, and 10% glycerol. Samples were concentrated using a Centricon-50 (Amicon) quantified by the Bradford protein dye assay (Bio-Rad) and aliquoted for storage at −80 °C. CyCyA and PCPC2 were purified as described previously (*5*).

Assay of VibF. Substrate-dependent ATP−PP_i exchange assays (100 μL) containing 10 nM VibF were performed as described previously (*4*). Heterocyclization/primary amine acylation assays (50 μL) contained 75 mM Tris, pH 7.5, 10 mM $MgCl_2$, 2 mM TCEP, 1 mM DHB, 5 μM holo-VibB, 10 mM L-Thr, 10 mM ATP, 0.5 μM holo-VibF, 2 μM VibE, and 1 mM DHB-NS. Heterocyclization/secondary amine acylation assays contained 75 mM Tris, pH 7.5, 10 mM $MgCl_2$, 2 mM TCEP, 1 mM DHB, 5 μM holo-VibB, 10 mM L-Thr, 10 mM ATP, 0.25 μM holo-VibF, 2 μM VibE, and 80 μM DHP-mOx-NS-DHB. Reactions were incubated at 30 °C for 5 and 50 min (primary amine acylation) or 3 and 30 min (secondary amine acylation) and analyzed by HPLC. Holo-VibB and VibF were generated as described previously (*4*).

Assay of CyCyA/PCPC2. Assays (50 μL) contained 75 mM Tris, pH 7.5, 10 mM $MgCl_2$, 2 mM TCEP, 1 mM DHB, 5 μM holo-VibB, 10 mM L-Thr, 10 mM ATP, 2 μM VibE, 80 μM DHP-mOx-NS-DHB, 4 μM CyCyA, and 10 μM PCPC2. Reactions were incubated at 30 °C for various amounts of time. Holo-PCPC2 was formed as described previously (*5*).

RESULTS

Design and Construction of VibF Mutant Expression Plasmids. Amino acid sequence alignments of both Cy and C domains have identified several regions of highly conserved sequence (*8*, *14*). In the Cy1 domain of yersiniabactin synthetase HMWP2, one such region with consensus DXXXXDXXS was examined by mutagenesis with the finding that both aspartic acid residues were essential to function while the serine was not (*15*). Alignment of Cy1 and Cy2 with this and other Cy domains identified D133A and D138A of Cy1 and D590A and D595A of Cy2 as the comparable residues (Figure 2). The catalytic core of C domains has the consensus sequence HHXXXD, in which the second histidine and the terminal aspartic acid residues have been shown to be critical, while the first histidine has not (*14*) (V. Bergendahl, personal communication). In VibF C1, the critical histidine is replaced with a glutamine; however, a functionally similar glutamate replaces the important aspartic acid, making E1552 a suitable candidate for a knock-out mutation. An alignment of VibF C2 with a representative sample of C domains clearly identifies H2084 as the important second histidine residue. Mutation of these residues to alanine was achieved by SOE mutagenesis using the primers of Table 1 and using the primer combinations of Table 2. The deletion mutant VibFΔCy1 was created by standard PCR and molecular biology techniques using the primer pair given in Table 2. All mutants were sequenced and found to contain only the desired alteration in the gene sequence.

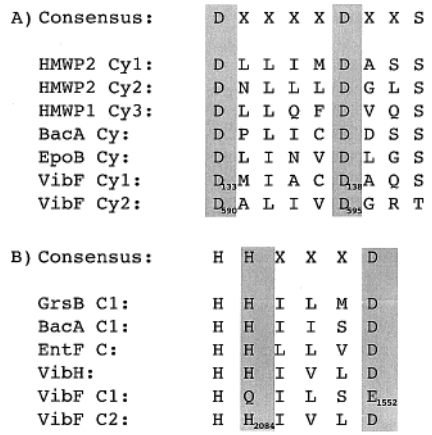


FIGURE 2: Section of an amino acid alignment of (A) the Cy domain core region DXXXXDXXS and (B) the C domain core region HHXXXD. Shading indicates amino acids that have been shown to be critical to function by biochemical assay. Numbers indicate the position in the VibF primary amino acid sequence.

Purification of VibF Mutant Enzymes and Assay by ATP−PP_i Exchange. *E. coli* BL21(DE3) hosting the various pVibF mutant plasmids were grown into stationary phase under the control of the T7 polymerase promoter without the addition of IPTG. Clarified lysates were batch-bound to nickel affinity chromatography resin and eluted with a step gradient of increasing concentrations of imidazole. The mutant proteins were found to have nickel affinity properties identical to those of the wild-type enzyme. All proteins were obtained in comparable yield and purity (Figure 3) with the exception of D138A and both double mutants. D138A was rapidly degraded during purification and could not be obtained in an intact form in any significant amount. Both of the double mutants, D133A/D590A and E1552A/H2084A, were obtained in reduced yield and purity (Figure 3). Migration on 9% SDS−PAGE of the wild-type and point mutant proteins was consistent with a calculated molecular mass of 271 kDa, as was migration of the 221 kDa ΔCy1 mutant.

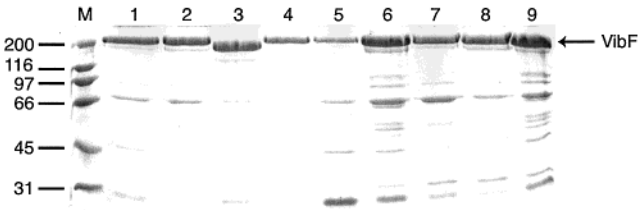


FIGURE 3: Coomassie blue stained 9% SDS−PAGE gel of purified VibF proteins used in this study. M: molecular weight standards. Lanes: 1, wild-type VibF; 2, D133A; 3, ΔCy1; 4, D590A; 5, D595A; 6, D133A/D590A; 7, E1552A; 8, H2084A; 9, E1552A/H2084A.

Table 3: VibF Adenylation Domain ATP−PP_i Exchange

VibF enzyme	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
wild type	1721 ± 77	0.6 ± 0.1	2.9×10^3
D133A	1708 ± 89	0.6 ± 0.1	2.8×10^3
ΔCy1	3885 ± 125	0.5 ± 0.1	7.8×10^3
D590A	1336 ± 53	0.5 ± 0.1	2.7×10^3
D595A	668 ± 22	0.8 ± 0.1	0.8×10^3
D133A/D590A	265 ± 25	1.3 ± 0.4	0.2×10^3
E1552A	1014 ± 46	1.0 ± 0.4	1.0×10^3
H2084A	1388 ± 74	1.1 ± 0.4	1.3×10^3
E1552A/H2084A	376 ± 30	1.1 ± 0.3	0.3×10^3

To determine the effect of the various mutations on the global function of VibF, we used the L-Thr-dependent ATP−PP_i exchange assay, a measure of A domain activity, as a diagnostic tool (Table 3). With the exception of the double mutants, all of the k_{cat} values of the mutant enzymes fell within a range of 2.5-fold difference from that of the wild type. The lower values of the double mutants, as much as 6.5-fold for D133A/D590A, are a reflection either of the lower purity of the samples or of a long-range perturbation in A domain activity by the mutations themselves or a combination of both. K_m values were very consistent, with a range of about 2-fold deviation from wild type.

Primary Amino Acylation Activity of VibF Mutant Enzymes. Both N_9 (primary) and N_5 (secondary) amine acylation reactions to make **2** and **1**, respectively, require (a) the self-acylation of the PCP domain with L-Thr by the A domain and (b) condensation of DHB with L-Thr-*S*-VibF and subsequent heterocyclization as prerequisite steps to amine acylation (Figure 1). Previous work with this system has demonstrated that self-acylation is not rate limiting (*5*), eliminating this source of masking of mutagenic-based reductions in either heterocyclization or amine acylation. The principal product of primary amino acylation by wild-type VibF is the expected N_9-acylated compound **2**; however, the uncyclized reaction product **4** is also observed with a partition ratio of about 0.1 (Figure 4A, Scheme 1B). All of the VibF mutants were examined for their ability to produce **2** and **4** and their rates of product formation compared to that of wild type (Table 4).

Both mutants aimed at removing Cy1 activity had essentially no effect on total product formation, with D133A turning over at 39 min^{-1} and ΔCy1 slightly lower at 30 min^{-1}. Both mutants, however, produced significantly more of the uncyclized product **4** than wild type, with partition ratio increases of 11-fold and >15000-fold for D133A and ΔCy1, respectively, implicating this domain in final processing to the heterocyclic DHP-mOx-*S*- group without a role in DHB-Thr condensation itself. Complementary to these

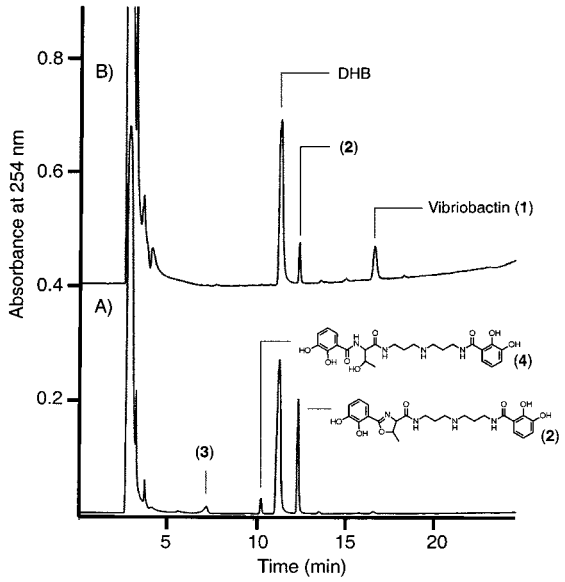


FIGURE 4: HPLC elution profiles of VibF products derived from (A) primary amine acylation, compounds **2** and **4**, and (B) secondary amine acylation, compound **1**.

Table 4: VibF Primary Amine Acylation Product Formation Rates

VibF enzyme	k_{obs} (min^{-1}) DHB-Thr-NS-DHB (**4**)	DHB-mOx-NS-DHB (**2**)	total product	product ratio (**4**:**2**)
wild type	3.7	36	40	0.1
D133A	20	19	39	1.1
ΔCy1	30	<0.02	30	>1500
D590A	0.4	3.9	3.9	0.1
D595A	<0.04	0.04	0.04	<1.0
D133A/D590A	2.0	1.5	3.5	1.3
E1552A	3.1	35	38	0.1
H2084A	0.2	4.5	4.5	0.04
E1552A/H2084A	0.3	5.8	5.8	0.05

observations was the effect of the mutations on the Cy2 domain. Both D590A and D595A suffered reductions in total product output (only 10-fold for the former but a convincing 1000-fold for the latter), with no observable effect on partition ratio, establishing Cy2 as the primary determinant in condensing activated DHB with L-Thr-*S*-VibF. A D133A/D590A double mutation maintained properties of both the individual mutants, turning over total products 10-fold less than the wild type, with a partition ratio increase of 13-fold. Taken together, these results suggest a surprising combined role for these two Cy domains in achieving what can typically be achieved by a single Cy domain in bacitracin, yersiniabactin, and epothilone synthetases. The secondary observation that in both Cy domains only a partial phenotype is observed in the first D mutation (of the DXXXXDXXS motif) suggests a diminished role for this residue in domain function, an observation that may have not been possible in the much less robust yersiniabactin system, with lowered signal-to-noise ratio (*15*).

The E1552A VibF mutant protein, an attempt to eliminate C1 activity, yielded a mutant with primary amine acylation activity identical to that of the wild type, suggesting that either this point mutation had no effect on the C1 domain or C1 had no role in this activity. This ambiguity was removed by the H2084A C2 mutant, which suffered a 10-fold reduction in product formation with essentially no

Table 5: VibF Secondary Amine Acylation Rates

VibF enzyme	k_{cat} (min^{-1})	VibF enzyme	k_{cat} (min^{-1})
wild type	41	D590A	5.3
D133A	23	D595A	0.09
ΔCy1	<0.04	E1552A	25
		H2084A	<0.04

change in the partition ratio of **4**:**2**. These results are consistent with the previous observation that VibF C2, when present in a PCP-C2 fragment, is capable of transferring DHP-mOx-*S*-PCP-C2 (formed by L-Thr, ATP, DHB-*S*-VibB, and the Cy1-Cy2-A fragment of VibF) to N_9 of DHB-NS supplied as a primary amine acceptor (*5*). Therefore, transfer of the DHP-mOx- group from the VibF PCP to the primary amine of DHB-NS is the province of the C2 domain, with no catalytic contribution by C1.

Secondary Amino Acylation Activity of VibF Mutant Enzymes. As with the primary amine acylation assay, VibF must form the DHP-mOx-*S*-VibF species prior to its transfer to N_5 of the intermediate, **2**, yielding the final product of the pathway, vibriobactin. Thus those mutations found to impede the DHB-Thr condensation (Cy2) or heterocyclization (Cy1) processes would be expected to exert their effects on vibriobactin formation rates, which were diagnostic of VibF secondary amine acylation activity (Figure 4B, Table 5). Consistent with this, then, was the 8-fold and 450-fold reduction in activity observed with the Cy2 mutants D590A and D595A, respectively, values that were reasonably proportionate with the reductions observed in primary amine acylation (10-fold and 1000-fold, respectively). The D133A and ΔCy1 mutants produced vibriobactin at 2-fold and >1000-fold reduced from wild type, with no evidence of any uncyclized threonine-containing derivatives of vibriobactin. This suggests that secondary amine acylation is intolerant of the uncyclized threonine donor and, consistent with this, vibriobactin formation by these two mutants occurs at rates equivalent to their formation of cyclized primary aminoacylated product.

While E1552A was modestly reduced at a rate of 1.6-fold relative to wild type, H2084A produced no detectable vibriobactin, a reduction of at least 1000-fold. This points to C2 as the sole catalytic domain responsible for secondary amine acylation, a somewhat remarkable assignment given that it is also the catalyst in primary amine acylation.

Vibriobactin Biosynthesis in the Absence of the C1 Domain. To firmly establish the role of C2 in both primary and secondary amine acylation and eliminate any role for the C1 domain, we tested the ability of a C1-deletion system to produce vibriobactin. The two VibF fragments Cy1-Cy2-A and PCP-C2 have been shown in previous studies to be capable of generating the intermediate **2** and are therefore known to be capable of primary amine acylation (*5*). Time-dependent formation of vibriobactin by these fragments, in the presence of DHB-*S*-VibB, L-Thr, ATP, and secondary amine acceptor **2**, is shown in Figure 5. Under the conditions tested, this system produced vibriobactin at a rate of ca. 30 μM/min and reached equilibrium after ca. 75% of the limiting substrate was utilized. The production of vibriobactin from Cy1-Cy2-A and PCP-C2 confirms that C2 is capable of both N_5 and N_9 acylation activities, while C1 is apparently dispensable.

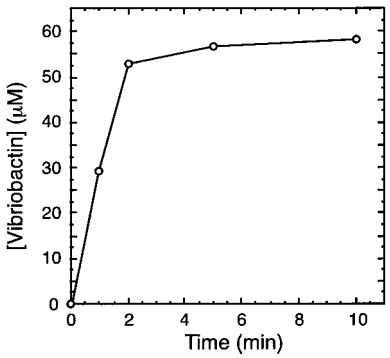


FIGURE 5: Time course of vibriobactin formation by the VibF fragments Cy1-Cy2-A and PCP-C2, when supplied with L-Thr, ATP, DHB-*S*-VibB, and compound **2**.

DISCUSSION

The *V. cholerae* iron chelator vibriobactin (**1**) is assembled from three molecules of 2,3-dihydroxybenzoic acid (DHB), two molecules of L-Thr, and one molecule of the symmetric triamine norspermidine, using a four-component nonribosomal peptide synthetase (NRPS) system. NRPS systems utilize a variety of enzymatic activities to facilitate the specific peptide and amide bond linkages they create, embodied by distinct domains which can exist independently or as part of a multidomain (and thus multicatalytic) protein synthetase. Those domains which function on the same amino acid residue of the peptide chain are usually found linked adjacent to one another and, as such, are referred to as "modules". This domain architecture is generally well conserved in the large multimodular systems that assemble the cyclic peptides of *Bacillus* and in the heptapeptide synthetases of *Streptomyces* (*16*, *17*). Siderophore NRPSs, however, tend to deviate from these structural paradigms and often present interesting arrangements of domains (*18*), domains which act on multiple modules (*19*) or domains which have evolved adaptive iterative activities (*20*), including those which assemble vibriobactin (*V. cholerae*), yersiniabactin (*Yersinia pestis*), and enterobactin (*E. coli*). The vibriobactin NRPS component VibF has six domains: two heterocyclization (Cy) domains, one A domain, one PCP domain, and two C domains. VibF is known to self-acylate its PCP with L-Thr, condense activated DHB onto the tethered L-Thr and subsequently heterocyclize to the (dihydroxyphenyl)methyloxazolinyl-*S*-VibF (DHP-mOx-*S*-VibF), and finally transfer this group twice to DHB-norspermidine (DHB-NS), once to the N_9 primary amine and then again to the N_5 secondary amine (*4*). The tandem Cy domains represent not only an unusual arrangement of domains but also a seemingly redundant catalytic activity, as other Cy domains that generate thiazoline and oxazoline rings are individually capable of all three required steps: condensation, cyclization, and dehydration (Scheme 1A). Furthermore, although two amine acylations are required, the placement of C1 between the usually adjacent A and PCP domains is another example of a deviation of the typical (-C-A-PCP-C-) NRPS architecture. We utilized mutagenesis to examine the roles of these domains in VibF catalysis. The basis for our choice of putative "knock-out" point mutations was previous work done on the conserved DXXXXDXXS motif of Cy domains, in which *Y. pestis* HMWP2 Cy1 was used as the specific target (*15*), and the conserved HHXXXD motif of C

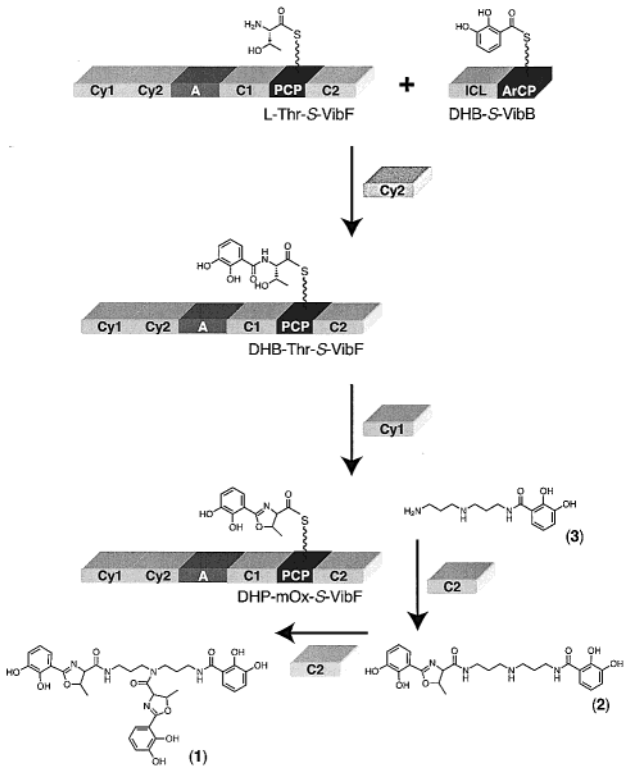


FIGURE 6: Catalytic mapping of VibF activities. Cy2 condenses activated DHB onto L-Thr-*S*-VibF, Cy1 processes DHB-Thr-*S*-VibF to the heterocyclic DHP-mOx-*S*-VibF, and C2 transfers this group twice, first to the N_9 of DHB-NS and then to N_5 of compound **2**.

domains, with the *Bacillus brevis* TycB C1 serving as the experimental subject (*14*). Both of the assays used to examine the effects of these mutations couple the prerequisite steps of self-acylation (A domain) and heterocycle formation (Cy domain) with the transfer of the cyclized DHP-mOx- group off of the VibF enzyme, in primary amine acylation to the N_9 amine of **3**, and in secondary amine acylation to the N_5 of **2** (Figure 1).

The mutants D133A and ΔCy1 both targeted the activity of the Cy1 domain, and in both assays resulted in the formation of a lower fraction of the heterocyclized DHP-mOx-*S*-VibF group for transfer, without exerting an effect on total flux in primary amine acylation in which DHB-Thr-*S*-VibF was found to be processed with comparable efficiency. This fortunate promiscuity in N_9-acylation allowed the observation that Cy1 has an insignificant role in the first step of the heterocycle process, condensation of DHB onto L-Thr-*S*-VibF, while having the required function of final processing to the heterocycle (Figure 6). Complementary to this was the effect on product formation observed in Cy2-targeted mutations, D590A and D595A, which reduced and eliminated product turnover, respectively, speaking to the role of Cy2 as the dedicated condensation domain that brings DHB onto L-Thr-*S*-VibF. The refusal of DHB-Thr-*S*-VibF as a substrate in secondary amine acylation allows us to eliminate even a slow conversion to DHP-mOx-*S*-VibF by Cy2 which may not be observable in the time scale of primary amine acylation. This division of labor by two Cy domains is unprecedented and raises the question of the evolutionary history of these tandem domains. One possibility is that they arose from a gene duplication event, and subsequently specialized to their specific function, with the additional cost of protein synthesis outweighed by benefit of siderophore production efficiency. While bioinformatic analysis does indicate that Cy2 and Cy1 deviate from other functionally characterized Cy domains, with an average of 17.4% and 21.2% similarity to six such domains, respectively, compared to an overall average of 25.7%, all of these other domains catalyze the formation of thiazoline rather than methyloxazoline heterocyclic rings (*4*, *6*, *8*, *21*). Cy2 does not align significantly better with C domains than with Cy domains, and in general the two groups of domains show very low homology in the range of 10−14% similarity. The tandem Cy domain feature of VibF suggests an evolutionary past in which derivatives of vibriobactin containing uncyclized L-Thr residues may have been the principal product, a not unrealistic scenario given that the six bidentate catechol hydroxyls provided by the aromatic rings alleviate the need of heterocyclic rings in iron coordination. In fact, siderophores from *E. coli* and *Bacillus subtilis* function as trimers of DHB-Ser and DHB-Thr, respectively (*22*, *23*).

In both attempts to eliminate Cy function, it was noted that mutation of the first Asp in the DXXXXDXXS motif was less potent than either the removal of the second Asp or the full domain deletion. This suggests a diminished role in function for this conserved residue, with a possible increased role for the second D, in both activities observed: condensation by Cy2 and the subsequent processing to heterocycle by Cy1. Both reactions presumably require similar mechanisms of catalysis, including an active site base to increase the nucleophilicity of an attacking amino (condensation) or alcohol (cyclization) group.

Previous studies of heterocyclization by VibF utilized small molecule substrate analogues, including L-Thr-*S*-pantetheine and L-Thr-*S*-*N*-acetylcysteamine thioesters, with the intent of probing acceptor substrate specificity independent of upstream A domain and downstream C domain selectivity. These substrates were in fact utilized, but only in condensation reactions with DHB-*S*-VibB, without further processing to heterocycle. That condensation and heterocycle formation are now understood to be catalyzed by separate domains with distinct active sites may have been a contributing factor to this discrimination of soluble substrate processing.

Primary amine acylation of DHB-NS to give **2** had been observed previously in a system using VibF fragments Cy1-Cy2-A and PCP-C2, and thus the complete retention of activity by E1552A (C1) versus the 9-fold loss incurred by H2084A (C2) was not surprising. The loss of only 1 order of magnitude in H2084A was less than anticipated. When the equivalent residue was mutated in the tyrocidine NRPS TycB C domain, a reduction in activity of at least 100-fold was observed (*14*), making this residue seem like a good candidate for an active site base in catalysis. C1 does not seem to contribute as the E1552A/H2084A double mutant was essentially equivalent to the H2084A mutant alone. This modest reduction in activity calls into question the role of the C2 domain histidine 2084 as a component of the catalytic machinery in, at the very least, the primary amine acylation activity of VibF.

The E1552A mutation had a similarly weak effect on secondary amine acylation activity, indicating no catalytic role for the C1 domain in VibF activity. Its lack of activity as a C domain is supported by bioinformatic analysis, in

which it is poorly aligned with other C domains and is missing critical residues in "core" regions. While robust turnover of both primary and secondary amine acylation products has been observed in the complete absence of C1, these experiments utilized VibF fragments that interacted in trans and do not eliminate a role for C1 in assisting domain−domain interactions in cis.

That VibF C2 was responsible for both primary and secondary amine acylation of DHB-NS and intermediate **2**, respectively, while unexpected, explained some previously observed behavior of VibF. Kinetic analysis of intermediate **2** utilization in vibriobactin formation demonstrated a strong substrate inhibition profile (*4*), which now appears likely the outcome of unproductive binding of this primary amine acylation product at higher concentrations. The use of a single C domain to catalyze two distinct acylation reactions has now been observed independently in a recent report describing the biosynthesis of myxochelin, in which DHB is added to both primary amines of a lysine residue by the system's sole C domain (*24*). VibF C2 adds to this new class of C domain and reveals that, if both acylations do, in fact, occur at the same active site, the data generated here suggest some significant differences in the way catalysis occurs in these two processes. First is the absolute refusal of uncyclized Thr in secondary but not in primary amine acylation, an unusual phenomenon given that the native donor in both these reactions (DHP-mOx-*S*-VibF) is unvaried. Second is the reductive effect of the H2084A mutation on turnover, only 9-fold in primary versus over 1000-fold in secondary amine acylation, clearly an argument for different modes of binding and/or catalysis in the two reactions. Given the difference in nucleophilicity of the secondary and primary amine substrates, perhaps it is not surprising that two different modes of catalysis are required, only that both are physically linked on a single NRPS domain. Detailed inhibitor studies may be required to elucidate the nature of and relationship between these two catalytic sites.

ACKNOWLEDGMENT

The authors thank Dr. Thomas A. Keating for insight in designing the mutagenesis strategy and reading of the manuscript, as well as Veit Bergendahl for personally communicated information on the tyrocidine NRPS TycB C1 domain.

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BI011852U