Vibriobactin Biosynthesis in *Vibrio cholerae*: VibH Is an Amide Synthase Homologous to Nonribosomal Peptide Synthetase Condensation Domains†

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Received July 14, 2000; Revised Manuscript Received September 20, 2000

ABSTRACT: The *Vibrio cholerae* siderophore vibriobactin is biosynthesized from three molecules of 2,3-dihydroxybenzoate (DHB), two molecules of L-threonine, and one of norspermidine. Of the four genes positively implicated in vibriobactin biosynthesis, we have here expressed, purified, and assayed the products of three: *vibE*, *vibB*, and *vibH*. All three are homologous to nonribosomal peptide synthetase (NRPS) domains: VibE is a 2,3-dihydroxybenzoate−adenosyl monophosphate ligase, VibB is a bifunctional isochorismate lyase−aryl carrier protein (ArCP), and VibH is a novel amide synthase that represents a free-standing condensation (C) domain. VibE and VibB are homologous to EntE and EntB from *Escherichia coli* enterobactin synthetase; VibE activates DHB as the acyl adenylate and then transfers it to the free thiol of the phosphopantetheine arm of VibB's ArCP domain. VibH then condenses this DHB thioester (the donor) with the small molecule norspermidine (the acceptor), forming N^1-(2,3-dihydroxybenzoyl)-norspermidine (DHB-NSPD) with a k_{cat} of 600 min^{-1} and a K_m for acyl-VibB of 0.88 μM and for norspermidine of 1.5 mM. Exclusive monoacylation of a primary amine of norspermidine was observed. VibH also tolerates DHB-acylated EntB and 1,7-diaminoheptane, octylamine, and hexylamine as substrates, albeit at lowered catalytic efficiencies. DHB-NSPD possesses one of three acylations required for mature vibriobactin, and its formation confirms VibH's role in vibriobactin biosynthesis. VibH is a unique NRPS condensation domain that acts upon an upstream carrier-protein-bound donor and a downstream amine, turning over a soluble amide product, in contrast to an archetypal NRPS-embedded C domain that condenses two carrier protein thioesters.

Many microbial pathogens have a demonstrated requirement for exogenous iron to maintain their growth and virulence. While iron may be present in large quantities in the host, the levels of free iron are generally very low, a product of the insolubility of Fe(III) and its tight sequestering by a variety of iron-binding proteins. To obtain iron from the host, many bacteria synthesize and secrete small molecule iron chelators (siderophores) and then selectively take up the siderophore and scavenged iron for intracellular utilization. Many of these iron-chelating virulence factors, including enterobactin from *Escherichia coli* (*1*), mycobactin from *Mycobacterium tuberculosis* (*2*), and yersiniabactin from *Yersinia pestis* (*3*), are peptidelike molecules that are nonetheless biosynthesized nonribosomally by large, multidomain enzymes termed nonribosomal peptide synthetases (NRPS). In NRPSs, the linear sequence of catalytic domains is responsible for assembly of the peptide product (*4*). Adenylation (A) domains catalyze monomer selection and activation (as the acyl adenylate) as well as covalent tethering of these monomers to the 4′-phosphopantetheine cofactor of a partner peptidyl carrier protein [PCP, also called thiolation (T) domain]. Coupling of two PCP-thioesterified monomers via an amide bond is carried out by condensation (C) domains (*5*). Extensive modification of the basic peptide product can occur both co- and postsynthetically and include epimerization, heterocyclization of cysteine and serine/threonine side chains, N- and C-methylation, macrocyclization, and glycosylation. These modifications are the province of specialized catalytic domains either inserted in the NRPS assembly line or present as separate, dedicated proteins. Interest in NRPS systems and their biosynthetic cousins, the polyketide synthases (PKS) (*6*), arises not only from the exquisite enzymatic machinery necessary for biosynthesis of complex natural products but also from the possibility of dissecting NRPS and PKS systems into sets of well-characterized, interchangeable catalytic reagents for the designed biosynthesis of natural-product-like molecules (*7*).

† This work has been supported by the National Institutes of Health (Grant AI042738 to C.T.W.). T.A.K. is a fellow of the Cancer Research Fund of the Damon Runyon−Walter Winchell Foundation (DRG-1483). C.G.M. is a postdoctoral fellow of the National Science and Engineering Research Council of Canada.

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[1] Abbreviations: NRPS, nonribosomal peptide synthetase; PKS, polyketide synthase; CoA, coenzyme A; A, adenylation domain; Cy, cyclization domain; TE, thioesterase; PPTase, phosphopantetheinyl transferase; P-pant, 4′-phosphopantetheine; PP_i, inorganic pyrophosphate; ICL, isochorismate lyase; ArCP, aryl carrier protein domain; PCP, peptidyl carrier protein domain; IPTG, isopropyl β-D-thiogalactopyranoside; PNP, calf spleen purine nucleoside phosphorylase; PP_iase, bakers' yeast inorganic pyrophosphatase; MESG, 2-amino-6-mercapto-7-methylpurine ribonucleoside; DTT, dithiothreitol; TCEP, tris(carboxyethyl)phosphine; TCA, trichloroacetic acid; DHB, 2,3-dihydroxybenzoate; NSPD, norspermidine; DAH, 1,7-diaminoheptane; DHB-NSPD, N^1-(2,3-dihydroxybenzoyl)norspermidine; DHB-DAH, *N*-(2,3-dihydroxybenzoyl)-1,7-diaminoheptane.

10.1021/bi001651a CCC: $19.00
Published on Web 11/22/2000

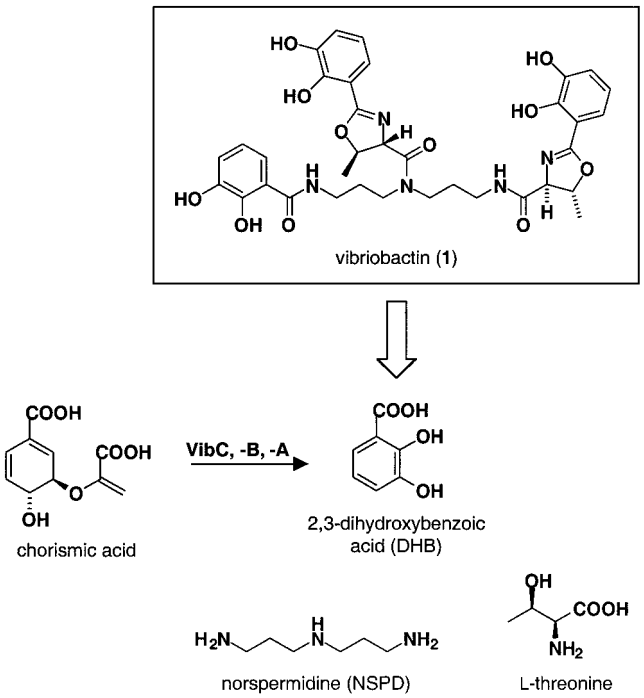

FIGURE 1: Retrobiosynthesis of the *Vibrio cholerae* iron chelator vibriobactin into 3 equiv of 2,3-dihydroxybenzoic acid (DHB), 2 equiv of L-threonine, and 1 equiv of norspermidine (NSPD). DHB is biosynthesized from chorismic acid by three enzymes, VibC, VibB, and VibA (*12*).

Outbreaks of cholera, caused by toxigenic *Vibrio cholerae*, a water-borne bacterium, cause an estimated 120 000 deaths annually worldwide (*8*). The major siderophore produced by *V. cholerae* was identified in 1984 as vibriobactin (**1**) (*9*). Since its isolation and structure elucidation, the genes responsible for the biosynthesis and transport of vibriobactin have been identified and localized to two gene clusters (*10*−*12*) separated by about 1 megabase on replicon I of *V. cholerae*'s two chromosomes (*13*). Four genes, *vibB*, *vibE*, *vibF*, and *vibH*, have been identified genetically as being required for vibriobactin biosynthesis (*10*, *11*) from 2,3-dihydroxybenzoic acid (DHB), L-threonine, and the unusual polyamine norspermidine (NSPD, Figure 1). The presence of NSPD as the central polyamine, in place of the more prevalent spermidine, is a hallmark of the *Vibrio* species (*14*). Several steps of NSPD biosynthesis from aspartate and L-2,4-diaminobutyrate have been characterized (*15*).

On the basis of homology to the *E. coli* enterobactin synthetase proteins EntE (*16*) and EntB (*17*), VibE and VibB have been proposed to function as a 2,3-DHB−adenosyl monophosphate ligase−aryl carrier protein (ArCP), respectively (*12*). Sequence analysis of VibH reveals its homology to the condensation domains of NRPSs, a domain heretofore seen and characterized only while embedded in larger NRPS proteins. We present here and in the accompanying paper (*40*) the first biochemical characterization of the vibriobactin synthetase culminating in the complete biosynthesis of vibriobactin in vitro; here, we focus on three of the four proteins, VibE, VibB, and VibH (Figure 2). VibE catalyzes activation of DHB as an acyl adenylate and then transfers this high-energy intermediate to the phosphopantetheinyl thiol of the ArCP component of VibB. VibH functions as a novel amide synthase that transfers this activated acyl group

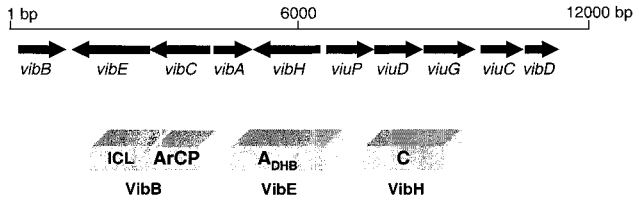

FIGURE 2: First gene cluster of vibriobactin synthetase (*11*, *12*) [for the second cluster, see the accompanying paper (*40*)]. VibB is a bifunctional protein: isochorismate lyase (ICL)−aryl carrier protein (ArCP). VibE is a 2,3-dihydroxybenzoate−AMP ligase homologous to the adenylate-forming superfamily of enzymes. VibH is an amide synthase homologous to nonribosomal peptide synthetase condensation (C) domains. VibC and VibA function in DHB biosynthesis, the Viu proteins comprise a vibriobactin transporter, and VibD is a phosphopantetheinyl transferase.

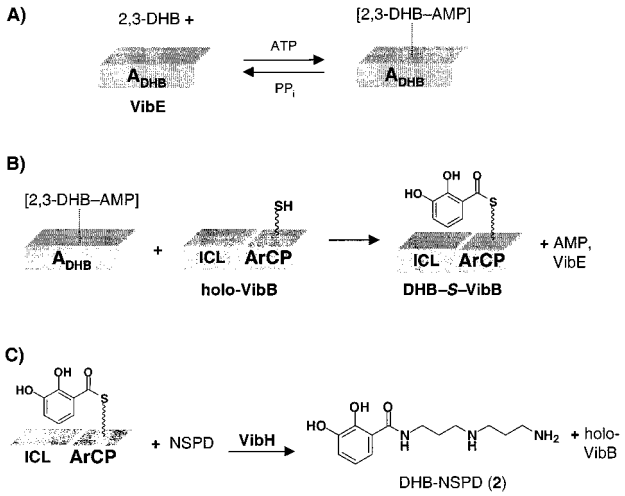

FIGURE 3: Activities of VibE, VibB, and VibH, culminating in the biosynthesis of N^1-(2,3-dihydroxybenzoyl) norspermidine (DHB-NSPD, 2). (A) VibE reversibly activates DHB as the sequestered acyl adenylate. (B) Holo-VibB is covalently acylated on its posttranslationally introduced phosphopantetheine thiol with DHB, transferred from VibE. (C) Condensation of this thioester with a primary amine of norspermidine is catalyzed by VibH, thus regenerating holo-VibB.

to a primary amine of norspermidine (Figure 3). This event is one of three amine acylations required to produce vibriobactin.

EXPERIMENTAL PROCEDURES

Materials and Methods. Standard recombinant DNA techniques and microbiological procedures were performed as described elsewhere (*18*). *V. cholerae* O395 genomic DNA was a gift from Professor Joan R. Butterton (Massachusetts General Hospital). Preliminary sequence data for *V. cholerae* O1, strain N16961, was obtained from The Institute for Genomic Research website at http://www.tigr.org. PCRs were carried out with *Pfu* polymerase according to its supplier's instructions (Stratagene). 2-Amino-6-mercapto-7-methylpurine ribonucleoside (MESG) was synthesized from 2-amino-6-chloropurine ribonucleoside according to published procedures (*19*). Phosphate-free, calf spleen purine nucleoside phosphorylase (PNP) and bakers' yeast inorganic pyrophosphatase (PP_iase) were purchased lyophilized from Sigma and stored as 0.01 unit/μL stocks in 75 mM Tris, pH 7.5, and 10 mM $MgCl_2$. 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). The phosphopantetheinyl transferases Sfp (*Bacillus subtilis*) (*20*) and EntD (*E. coli*) (*21*) have been described previously, as have adenylation enzyme EntE (*16*) and VibB homologue EntB (*22*). Protein concentrations were determined from the predicted molar extinction coefficient (ϵ) and absorbance at 280 nm. HPLC analyses were performed on a Beckman System Gold equipped with a Vydac small-pore C18 reverse-phase analytical column. Detection was at 254 nm, with mobile phase A, 0.08% trifluoroacetic acid (TFA) in water, and mobile phase B, 0.08% TFA in acetonitrile. At a flow rate of 1 mL/min, a linear gradient was maintained from 10% to 100% B over 23 min. Large-scale enzymatic reactions were purified by reverse-phase preparative HPLC with the mobile phases listed above. ^{1}H NMR spectra were acquired with a Varian 200 MHz spectrometer and referenced to residual solvent resonances. Coupling constants are listed in hertz. Positive-ion detection matrix-assisted laser desorption−ionization (MALDI) mass spectrometry was performed at either Harvard Medical School or the Dana-Farber Cancer Institute.

Cloning of vibB, vibE, and vibH. The genes *vibB*, *vibE*, and *vibH* were amplified by PCR from *V. cholerae* O395 genomic DNA with the following primer pairs (VibB1, 5′-ATGTACT**CAT**ATGGCTATTCCTAAAATCGC-3′, and VibB2, 5′-CTCGTTC**TCGAG**CGCAGAGCAAGGTTGATGT-3′; VibE1, 5′-GGAGATA**CAT**ATGAC**C**ACCGATTTTACCCC-3′, and VibE2, 5′-ACATTG**GTCGAC**CGACATTAACGCTAAGCC-3′; and VibH1, 5′-TATCGTG**CA**TATGTTATTGGCTCAAAAGCC-3′, and VibH2, 5′-TTCATCT**CGAG**ACTGGCGATAGCCCGTG-3′; restriction sites underlined, mutations in boldface type). The *vibB* and *vibH* amplicons were digested with *Nde*I and *Xho*I, the *vibE* amplicon was digested with *Nde*I and *Sal*I, and each was ligated to the corresponding sites of pET29b (Novagen). The resulting plasmids, pVibB, pVibE, and pVibH, each express a translational fusion of a C-terminal hexahistidine tag with the respective protein. The plasmids were used to transform *E. coli* DH5α and then transferred to strain BL21(DE3) for expression.

Expression and Purification of VibB, VibE, and VibH. For overproduction of VibB and VibE, BL21(DE3) strains carrying pVibB or pVibE were cultivated (2 × 1 L) with shaking (250 rpm) at 37 °C in Luria−Bertani broth containing 40 μg/mL kanamycin. When the culture reached an OD_{600} of 0.8, the temperature was lowered to 30 °C. Cultures were induced with 400 μM IPTG, and shaking proceeded for 4 h. For overproduction of VibH, which was insoluble when expressed after IPTG induction at 30 °C, 6 × 1 L cultures of BL21(DE3) pVibH were shaken (250 rpm) for 18 h at 25 °C without induction. For purification of all three proteins, cells were harvested by centrifugation (10 min at 2000*g*) and resuspended in 20 mM Tris, 500 mM NaCl, and 5 mM imidazole, pH 8.0 (buffer A). Following resuspension, cells were disrupted by two passages through a French pressure cell, and the lysate was clarified by centrifugation (30 min at 95000*g*). The proteins were purified by nickel chelate chromatography on Ni−NTA Superflow resin (Qiagen) as described previously (*23*). VibH underwent a second purification step on an 8 mL Source 15Q anion-exchange column (Pharmacia), also as described previously (*23*). Eluted proteins were dialyzed against 25 mM Tris, 50 mM NaCl, 1 mM DTT, and 10% glycerol, pH 8.0, and then flash-frozen and stored at −80 °C.

ATP−PP$_i$ Exchange Assay for VibE Substrate Specificity. Reactions (100 μL) contained 75 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 3.5 mM ATP, 1 mM sodium pyrophosphate (0.18 μCi), 30−300 nM VibE, and varying concentrations of substrate. Reactions were incubated at 30 °C for 10 min and quenched by addition of 1.6% (w/v) activated charcoal, 200 mM tetrasodium pyrophosphate, and 3.5% perchloric acid in water. The charcoal was pelleted by centrifugation and washed twice with 200 mM $Na_4P_2O_7$ and 3.5% perchloric acid, and the bound radioactivity was measured by liquid scintillation counting.

Analysis of Phosphopantetheinylation of the ArCP Domain of VibB with [^{3}H]Coenzyme A. Reactions (20 μL) to determine the required time for full phosphopantetheinylation of VibB contained 75 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.5 M NaCl, 1 mM TCEP, 40 μM VibB, 82 μM [^{3}H]coenzyme A (0.21 μCi), and 3 μM PPTase and were incubated for various times at 30 °C. Reaction products were precipitated by addition of 0.2 mL of 10% trichloroacetic acid (TCA) and 50 μg of BSA and pelleted by centrifugation. Pellets were washed twice with 10% TCA, redissolved in 180 μL of formic acid, and submitted for liquid scintillation counting.

Analysis of Covalent [^{14}C]Salicylation of Holo-VibB and Holo-EntB by VibE. Reactions (50 μL) contained 75 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.5 M NaCl, 5 mM TCEP, 5 mM ATP, 75 μM salicylate (0.21 μCi), 60 nM VibE, and varying concentrations of holo-VibB. Reactions were incubated at 30 °C for 6 min before TCA precipitation as above. Holo-VibB was formed in 500 μL reactions for 60 min containing 75 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.5 M NaCl, 1 mM TCEP, 100 μM CoA, 6 μM apo-VibB, and 300 nM Sfp. Reactions with holo-EntB as substrate were identical except for the addition of 120 nM VibE.

Analysis of Covalent 2,3-Dihydroxybenzoylation of Holo-VibB by VibE. A coupled, continuous, spectrophotometric assay for inorganic pyrophosphate was employed (*24*, *25*). Reactions were carried out at 30 °C in 200 μL total volume in a quartz cuvette that contained 75 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.5 M NaCl, 5 mM TCEP, 200 μM MESG, 0.2 unit of PNP, 0.2 unit of PP$_i$ase, 5 mM ATP, 20 μM DHB, 100 nM VibE, and varying concentrations of holo-VibB. Reactions were initiated by addition of the amino acid substrate after a 10 min incubation to allow the PP$_i$ase/PNP/MESG couple to remove any contaminating PP_i or P_i. A spectrophotometer monitored absorbance at 360 nm and computed rates of A_{360} increase. Substrate-specific pyrophosphate release was measured by subtracting the rate of A_{360} increase before amino acid addition from the ΔA_{360} rate after addition. This net rate was converted to reaction velocity through the molar extinction coefficient of MESG [ϵ = 17 600 $(M\cdot cm)^{-1}$].

Amide Synthase Activity of VibH with Various Amine Acceptors. To obtain kinetic data for the production of N^1-(2,3-dihydroxybenzoyl)norspermidine (DHB-NSPD, **2**) by VibB, VibE, and VibH, 100 μL reactions contained 75 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM TCEP, 100 μM coenzyme A, 5 mM DHB, 5 mM ATP, 0.3 μM Sfp, 5 μM VibB, 2.5 μM VibE, 0.3 μM VibH, and varying concentrations of norspermidine. Reactions were incubated for 40 min

at room temperature to allow for phosphopantetheinylation of VibB by Sfp before initiation by NSPD addition. Reactions proceeded at 25 °C for 10 min before they were quenched with 900 μL of cold MeOH. Mixtures were then centrifuged at 4 °C for 30 min, and the supernatant was subsequently removed and evaporated to dryness. After resuspension in 30% CH_3CN, the samples were analyzed by HPLC. Kinetic data for production of *N*-(2,3-dihydroxybenzoyl)-1,7-diaminoheptane (DHB-DAH, **3**) was obtained similarly, except that reactions proceeded for 30 min. Octylamine and hexylamine were examined similarly, except for reaction time (15 and 30 min, respectively), and reactions were quenched with 20 μL of 8.5% H_3PO_4 and extracted with ethyl acetate. The organic layer was removed and evaporated to dryness, and the residue was resuspended in 30% CH_3CN for HPLC analysis. The identities of the acylated amine products were verified by mass spectrometry of HPLC-purified samples and by ^{1}H NMR (DHB-NSPD and DHB-DAH) or by HPLC analysis of a synthetic standard (octyl 2,3-dihydroxybenzamide **4**). To quantify the amounts of products formed, a calibration curve based on HPLC peak area was generated from injection of known amounts of synthetic octyl 2,3-dihydroxybenzamide.

Kinetics of Amide Synthase Activity of VibH with VibB and EntB. To obtain saturation kinetics for VibB in the production of **2**, 100 μL reactions contained 75 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM TCEP, 50 μM CoA, 2 mM 2,3-DHB, 2 mM NSPD, 3 mM ATP, 0.26 μM Sfp, 1.5 μM VibE or 1 μM EntE, 40 nM VibH, and varying concentrations of VibB or EntB. Reactions proceeded for 15 min after phosphopantetheinylation before workup as above.

Synthesis of Octyl 2,3-Dihydroxybenzamide as a Standard. 2,3-DHB (1 mmol, 154 mg) and 1-hydroxybenzotriazole hydrate (1 mmol, 153 mg) were dissolved in 1.3 mL of dry THF. Dicyclohexylcarbodiimide (1 mmol, 206 mg) dissolved in 1 mL of dry THF was added dropwise. After stirring at room temperature for 15 min, octylamine (1 mmol, 165 μL) was added and the mixture was stirred for an additional 1 h. Ethyl acetate (2 mL) was then added and the reaction was filtered to remove dicyclohexylurea. After evaporation of solvent under reduced pressure, the residue was taken up in 8 mL of ethyl acetate and washed with 4 mL of saturated sodium bicarbonate solution. The organic portion was dried over sodium sulfate and evaporated to dryness. The residue was purified by silica gel column chromatography with chloroform as eluant. Yield = 186 mg, 70%.

RESULTS

Cloning and Expression of vibB, vibE, and vibH and Purification of Gene Products. The *vibB*, *vibE*, and *vibH* genes were amplified from *V. cholerae* serotype O395 genomic DNA and cloned into C-terminal His-tag vectors for overexpression. Sequencing of pVibB (two independent PCR amplifications) revealed three nucleotide changes, all silent, from the sequence in GenBank (strain Lou15, accession no. U52150). Sequencing of pVibE (two independent PCR amplifications) revealed a single nucleotide change that alters the Gly199 in the GenBank sequence to Asp. Sequencing of pVibH revealed a nucleotide change that alters Leu285 in the GenBank sequence to Ser. However, TIGR's sequencing project of *V. cholerae* serotype O1 has found Ser285 in VibH (*26*). These revised sequences for *vibB*, *vibE*, and *vibH* have been deposited in GenBank (accession numbers AF287252, AF287253, and AF287254).

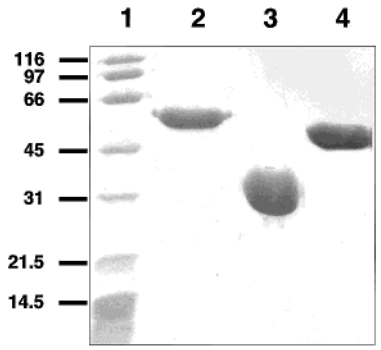

FIGURE 4: Coomassie-blue stained 12% SDS−PAGE gel of purified Vib proteins used in this study. Lane 1, molecular weight standards; lane 2, VibE; lane 3, VibB; lane 4, VibH.

Table 1: Kinetics of Substrate-Dependent ATP−PP_i Exchange Catalyzed by VibE and Homologues EntE and YbtE

enzyme	substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1}M^{-1}$)
VibE	2,3-DHB	138 ± 1	0.46 ± 0.01	5×10^6
	salicylate	29.3 ± 0.2	16 ± 1	3×10^4
EntE[a]	2,3-DHB	330	2.7	2×10^6
	salicylate	150	91	3×10^4
YbtE[b]	2,3-DHB	14	400	6×10^2
	salicylate	230	4.6	8×10^5

[a] See ref *16*. [b] See ref *27*.

Standard inducible *E. coli* overexpression resulted in 82 mg/L purified VibB and 27 mg/L purified VibE after nickel-affinity purification. VibH produced after IPTG induction and growth at 30 °C was insoluble, while growth at 25 °C for 18 h without induction yielded 20 mg/L VibH after successive nickel-affinity and anion-exchange purification steps. Results of purification are shown in Figure 4.

ATP−PP_i Exchange Assay for VibE Substrate Specificity. Results for the substrate-dependent ATP−PP_i exchange reaction catalyzed by VibE are shown in Table 1. Michaelis−Menten saturation kinetics were observed for both the natural substrate 2,3-DHB and its deshydroxyl analogue salicylic acid. VibE discriminated between the two by a factor of 150 in catalytic efficiency. These kinetic parameters are quantitatively similar to those measured previously for adenylation homologues EntE (enterobactin synthetase) (*16*) and YbtE (yersiniabactin synthetase) (*27*).

Analysis of Phosphopantetheinylation of the ArCP Domain of VibB with [^{3}H]Coenzyme A. Two different phosphopantetheinyl transferases, Sfp and EntD, were used to covalently attach a 4′-phosphopantetheine arm derived from coenzyme A to Ser248 of VibB [the presumed native PPTase, VibD (*12*), was not used in this study]. Results are shown in Figure 5. The stoichiometry of phosphopantetheinylation was measured by incorporation of tritium label from [^{3}H]CoA and was referenced to the PPTase/ArCP pair EntD/EntB from *E. coli*. When PPTase was omitted, no phosphopantetheine was incorporated. EntD was very inefficient in modifying VibB, even though EntD has been shown to accept several non-Ent ArCPs and PCPs previously (*27*). However, Sfp, the promiscuous PPTase from the surfactin synthetase cluster of *Bacillus subtilis* (*20*), was able to form holo-VibB in good

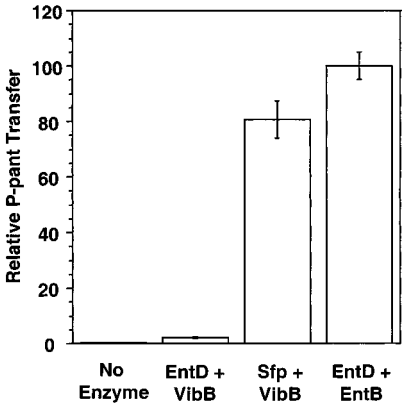

FIGURE 5: Phosphopantetheinylation of VibB with [^{3}H]coenzyme A. Relative level of P-pant incorporation into VibB with phosphopantetheinyl transferases EntD and Sfp is standardized to that with the EntD/EntB pair.

relative stoichiometry (80%), as compared to the well-characterized EntD/EntB pair. A time course of holo-VibB formation by Sfp was also performed; final stoichiometry in Figure 5 was reached after 60 min.

Analysis of Covalent Thioesterification of Holo-VibB and Holo-EntB by VibE. Because radiolabeled 2,3-DHB is not commercially available, a coupled assay for pyrophosphate was employed to obtain kinetics of transfer of activated DHB from VibE to the phosphopantetheine arm of holo-VibB (Figure 3). The results are plotted in Figure 6A. Substrate inhibition by holo-VibB is observed, with a maximum initial velocity for VibE of 19 min^{-1} and an apparent K_m of 3 μM. To measure the kinetics of analogous salicylate transfer, the incorporation of [^{14}C]salicylate into precipitated VibB or EntB was measured by scintillation counting. The results are plotted in Figure 6B. Again, the reaction suffers from substrate inhibition above about 5 μM VibB, and thus saturation kinetics could not be determined. The maximum observed initial velocity is 5 min^{-1}; the concentration of VibB at which half this velocity is reached (K_m) is 2 μM. In contrast, VibE salicylated the homologue EntB with k_{cat} = 4.0 min^{-1} and K_m = 18.6 μM. Thus, in the second step catalyzed by VibE, the thioesterification of acyl adenylate, changing from the natural substrate DHB to salicylate reduces the maximum velocity by 4-fold with no impact upon holo-VibB K_m, whereas substitution of EntB for VibB reduces k_{cat}/K_m by 12-fold.

Analysis of Acylated Amine Products of VibH and 2,3-DHB−S−VibB. With acylated VibB as a donor substrate for the amide synthase VibH, we investigated potential acceptor substrate nucleophiles. Along with the presumed native substrate norspermidine, we tested 1,7-diaminoheptane (DAH), octylamine, hexylamine, and 1-octanol. By HPLC, we observed single new products formed for all of these acceptor substrates except 1-octanol. To identify these products, they were purified by HPLC and analyzed by MALDI-TOF mass spectrometry. The results are shown in Figure 7. All gave masses consistent with monoacylation with 2,3-DHB. For NSPD and DAH, no di- or triacylation was observed. The HPLC retention time of **4** matched that of a synthetic standard. Additionally, **2** and **3** were characterized by ^{1}H NMR. Asymmetry in the NMR spectrum of **2** established that acylation occurred at a primary amine, not the secondary amine. Uncatalyzed rates of acylated amine formation were

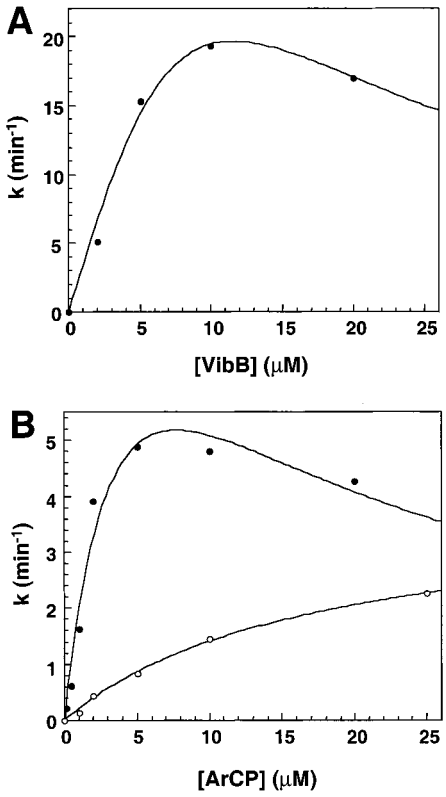

FIGURE 6: Kinetics of ATP- and substrate-dependent ArCP acylation by VibE. (A) Kinetics of DHB transfer from VibE to holo-VibB, measured by a continuous spectrophotometric assay for pyrophosphate. The maximum k measured for this transfer is 20 min^{-1} at 10 μM VibB. Substrate inhibition is seen at higher VibB concentrations. Half-maximal velocity (K_m) is reached at 3 μM VibB. (B) Kinetics of salicylate transfer to VibB (●) and EntB (○), measured by covalent incorporation of [^{14}C]salicylate into the holo-ArCP. Data were fit with the Michaelis−Menten equation with substrate inhibition (VibB) or without substrate inhibition (EntB). Maximum k for transfer of salicylate to VibB is 5 min^{-1}, with half this velocity reached at 2 μM VibB. For EntB, k_{cat} = 4.0 ± 0.2 min^{-1} and K_m = 18.6 ± 1.7 μM.

examined by omitting VibH in reactions containing VibE and VibB, and in reactions containing only VibE. No product was seen with acceptor substrates NSPD and 1,7-DAH in time courses out to 100 min. DHB-NSPD **2** (CD_3OD): δ 7.22 (dd, 1H, J = 8.0 and 1.4 Hz), 6.94 (dd, 1H, J = 7.8 and 1.6 Hz), 6.73 (t, 1H, J = 8.1 Hz), 3.53 (t, 2H, J = 6.4 Hz), 3.09 (m, 6H), and 2.04 (m, 4H). DHB-DAH **3** (CD_3CN): δ 7.42 (br s, 1H), 7.13 (dd, 1H, J = 8.0 and 1.4 Hz), 6.99 (dd, 1H, J = 8.0 and 1.4 Hz), 6.76 (t, 1H, J = 7.9 Hz), 3.38 (q, 2H, J = 6.2 Hz), 2.95 (m, 2H), 1.63 (m, 4H), and 1.37 (m, 6H).

Kinetics of 2,3-DHB−S−VibB and 2,3-DHB−S−EntB as Substrates for VibH. To obtain kinetics for VibB and EntB ArCP domains as donor substrates for VibH, acceptor NSPD was held at 2 mM while holo-VibB and holo-EntB concentrations were varied. Results are shown in Figure 8 and Table 2. Saturation kinetics for VibB were observed and half-maximal velocity (K_m) was seen at 0.88 μM holo-VibB, with k_{cat} = 600 min^{-1}. By contrast, the homologue EntB saturated with k_{cat} = 232 min^{-1} and K_m = 6 μM. This represents a 2.5-fold decrease in k_{cat} and a 7-fold increase in K_m relative to VibB.

Kinetics of Amine Acylation by VibH. To obtain kinetics for the four amine acceptor substrates for VibH, holo-VibB

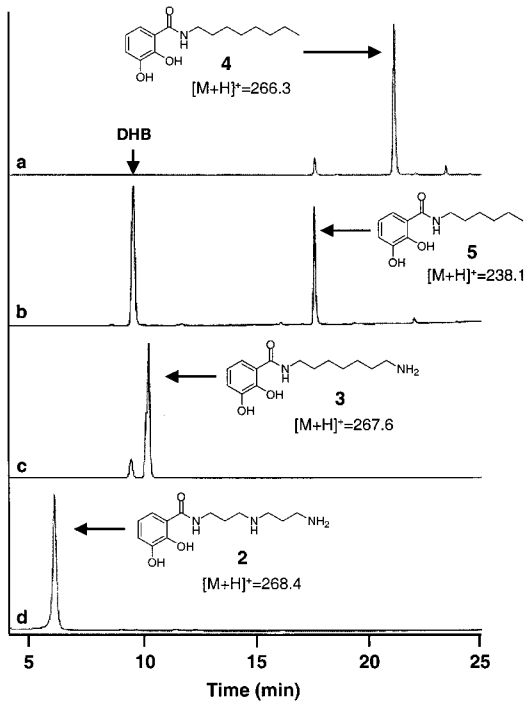

FIGURE 7: HPLC elution profiles of amides formed by VibH with donor DHB−*S*−VibB and four different amine acceptors. Reactions contained (a) octylamine, (b) hexylamine, (c) 1,7-diaminoheptane, and (d) norspermidine. Unreacted DHB is present in several reactions. Reactions products are indicated with arrows, and the mass of the $[M + H]^+$ ion obtained by MALDI-TOF analysis is listed below each compound.

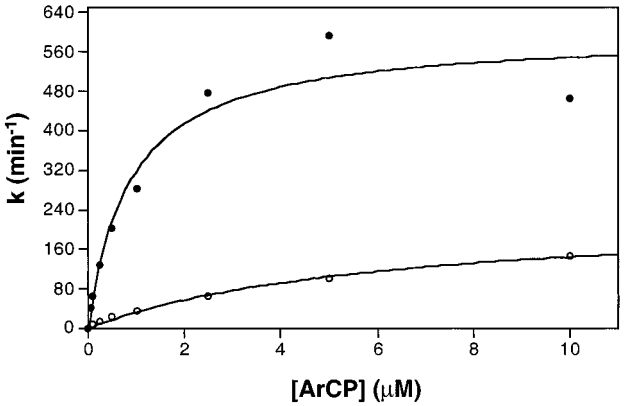

FIGURE 8: Saturation of VibH amide synthase activity with holo-VibB (●) and holo-EntB (○). VibH was incubated for fixed times with VibE or EntE, DHB, NSPD, ATP, and increasing concentrations of either VibB or EntB. Both sets of data were fit the Michaelis−Menten equation, although it is possible that mild substrate inhibition is present at higher concentrations of VibB. See Table 2 for apparent kinetic constants.

was held at 5 μM while the amine concentrations were varied. Results are shown in Figure 9 and in Table 2. Norspermidine showed substrate inhibition above 3 mM. The maximum velocity measured was 430 min^{-1}, with half-maximal velocity (K_m) reached at 1.5 ± 1 mM. 1,7-Diaminoheptane was better behaved, yielding saturation kinetics with k_{cat} = 697 min^{-1} and K_m = 6.2 mM. Both octylamine and hexylamine could not be saturated as substrates, with octylamine precipitating all enzymes above 30 mM and hexylamine reaching 192 mM without saturation of velocity. Thus, the kinetic constants listed in Table 2 are the highest measured values. Catalytic efficiencies (k_{cat}/K_m) show clear discrimination among the four amine substrates, with norspermidine 2-fold higher than 1,7-diaminoheptane and with octylamine and hexylamine down 60-fold and 700-fold, respectively.

Table 2: Kinetic Constants for Alkylamine Acceptors and Holo-ArCP Donors with VibH

substrate	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})
norspermidine	≥430	1.5 ± 1	≥4.8 × 10^3
1,7-diaminoheptane	697 ± 6	6.2 ± 0.2	1.9 × 10^3
octylamine	≥157	≥30	≥87
hexylamine	≥84	≥192	≥7
substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M^{-1})
VibB	600 ± 50	0.88 ± 0.25	1.1 × 10^7
EntB	232 ± 18	6 ± 1	6.4 × 10^5

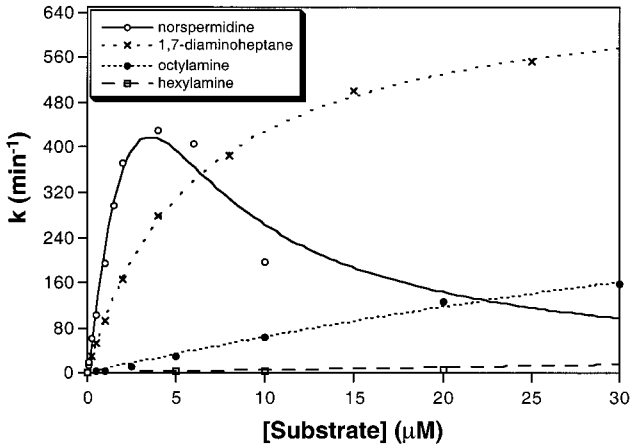

FIGURE 9: Concentration-dependent VibH amide synthase activity with various amine nucleophiles. VibH was incubated with VibE, holo-VibB, ATP, DHB, and increasing concentrations of four amines. Norspermidine suffered substrate inhibition above ~5 mM, and octylamine >30 mM caused protein precipitation. Saturation kinetics were not observed for hexylamine out to 192 mM. Norspermidine data were fit to the substrate inhibition equation, while the data from the other amines were fit to the Michaelis−Menten equation. See Table 2 for apparent kinetic constants.

DISCUSSION

Of the four genes positively identified in vibriobactin biosynthesis in *V. cholerae*, *vibE*, *vibB*, *vibH*, and *vibF*, two gene products, VibE and VibB, are homologous to the well-characterized components of the *E. coli* siderophore-forming enterobactin synthetase EntE and EntB (*12*). EntB is a bifunctional enzyme; the N-terminal 200 residues comprise an isochorismate lyase that catalyzes the second to last step in 2,3-DHB biosynthesis from chromate, while the C-terminal 80−100 residues have been identified as an aryl carrier protein (ArCP), which is posttranslationally modified by addition of a CoA-derived 4′-phosphopantetheine prosthetic arm by the phosphopantetheinyl transferase EntD (*22*). By homology, VibB also appears to be a bifunctional isochorismate lyase−ArCP (44% identity, 61% homology to EntB) and is similar to two other putative bifunctional enzymes, VenB from vulnibactin synthetase of *Vibrio vulnificus* (*28*) (56% identity, 74% homology), and DhbB of *B. subtilis* (*29*) (46% identity, 63% homology). All four of these proteins possess the core motif GLDSxR in their ArCP domains, the conserved serine hydroxyl side chain being the point of attachment of the phosphopantetheine.

VibE is homologous to EntE (47% identity, 65% homology), a 2,3-dihydroxybenzoate−AMP ligase responsible for activating 2,3-DHB as the acyl adenylate in an ATP-dependent manner and for transferring this activated species to the free P-pant thiol of holo-EntB. 2,3-DHB−*S*−P-pant-EntB then acts as the donor carrier protein to the peptide synthetase EntF for enterobactin biosynthesis. VibE is thus a member of the large family of adenylation (A) enzymes of peptide synthetases, which activate monomeric amino and aryl acids and thioesterify them on the P-pant thiols of carrier protein domains. While most A domains are embedded in multidomain NRPS proteins, several other homologous free-standing adenylation enzymes that activate aryl acids include DhbE from *B. subtilis* (*29*) (52% identity, 69% homology), PchD from pyochelin synthetase of *Pseudomonas aeruginosa* (*30*) (50% identity, 65% homology), and YbtE from yersiniabactin synthetase of *Y. pestis* (*27*) (45% identity, 60% homology).

VibH is a 436 residue, 49.8 kDa protein with homology to condensation (C) domains of NRPSs. C domains catalyze the amide-bond forming step of nonribosomal peptide synthesis between two carrier-protein-thioesterified acyl substrates: an upstream donor and a downstream acceptor, which is typically an amino acid (*31*). With few exceptions [e.g., the PapA5 protein putatively involved in mycocerosic acid biosynthesis in *M. tuberculosis* (*32*, *33*)], C domains are exclusively embedded within larger multidomain NRPSs. We present here the first biochemical characterization of VibH as a novel amide synthase that couples 2,3-DHB thioesterified to holo-VibB to a primary amine of norspermidine as well as to several other primary amines. This acylation event, which yields N^1-(2,3-dihydroxybenzoyl)norspermidine 2, is one of three required to produce vibriobactin.

After expression and purification of VibB, we began by attempting in vitro phosphopantetheinylation to convert the inactive apo-VibB to the holo form competent for acylation. Surprisingly, despite the homology of VibB to EntB and the demonstrated ability of EntD to recognize heterologous carrier protein domains, the enterobactin PPTase EntD was not able to efficiently modify VibB. However, the *B. subtilis* PPTase Sfp did recognize and modify VibB, as judged by covalent incorporation of radiolabel from [^{3}H]CoA (Figure 5). Sfp was used in all subsequent reactions involving VibB, and a preincubation period was used to allow for conversion to the holo form prior to acylation reactions.

VibE was characterized according to its two-step catalytic process. In the first step, substrate is reversibly activated with ATP to form the acyl adenylate. The classic substrate-dependent ATP−PP_i exchange reaction verified the robust activation of 2,3-DHB, the predicted natural substrate for VibE based on the vibriobactin structure (Table 1). The close structural homologue salicylic acid was also activated, albeit 150-fold worse in catalytic efficiency. For comparison, data for EntE and YbtE, which activate 2,3-DHB and salicylate, respectively, are included in Table 1. As can be seen, VibE is comparable to these adenylation enzymes.

The second step of adenylation enzyme activity is transfer to a holo-carrier protein domain (Figure 3). Based on the vibriobactin gene cluster and the enterobactin synthetase precedent, this partner is holo-VibB. Transfer of activated substrate from VibE to VibB was verified and characterized in two ways. In a coupled assay for pyrophosphate production, 2,3-DHB transfer to VibB displayed substrate inhibition at high concentrations of VibB. A maximum transfer rate of 20 min^{-1} was measured at 10 μM VibB, with half this rate (K_m) reached at 3 μM (Figure 6a). When [^{14}C]salicylate was used to measure transfer of this substrate, the maximum rate was down 4-fold (5 min^{-1}) with the VibB K_m essentially unchanged at 2 μM. Although substrate inhibition again made saturation kinetics impossible to determine, we conclude that both 2,3-DHB and salicylate are transferred to VibB, salicylate only 4-fold less efficiently. We also evaluated the ArCP EntB as an acceptor substrate for VibE in the radiolabeled salicylation assay. Catalytic efficiency (k_{cat}/K_m) was reduced by only 12-fold relative to VibB, demonstrating that the close sequence homology of VibB and EntB is reflected in their similar behavior as substrates and indicating the clear role of the ArCP in presentation of the phosphopantetheine thiol and recognition by VibE.

With VibE and VibB characterized and shown competent for production of DHB−*S*−VibB (Figure 3), we assayed VibH for transfer of activated DHB from VibB to a variety of nucleophiles. Four primary amines were successful acceptor substrates: norspermidine, 1,7-diaminoheptane, octylamine, and hexylamine, while 1-octanol was not. The kinetics for these substrates are shown in Figure 9 and Table 2. Classical saturation kinetics were not obtainable for three of the substrates (NSPD suffered from substrate inhibition above 5 mM, octylamine precipitated all proteins above 30 mM, and hexylamine at 192 mM did not saturate amide formation). Products in all cases were the result of monoacylation of a primary amine, and were verified by mass spectrometry and by either NMR (**2** and **3**) or HPLC analysis of synthetic standard (**4**).

An examination of the kinetic constants for the four substrates (Table 2) shows NSPD to be the best substrate (at low concentrations) with a maximum k of 430 min^{-1} and a K_m of 1.5 mM. The ~2.5-fold decrease in catalytic efficiency experienced by 1,7-DAH may reflect the importance of the central, secondary amine in NSPD in recognition by VibH, despite this amine not being involved in the transfer event. Even more starkly, octylamine, which is missing both a primary and the secondary amine of NSPD but has the same number of backbone atoms, is down some 50-fold in catalytic efficiency. Thus, the distal amine of NSPD must also play an important role in binding of and transfer to the reacting amine. Shortening the alkyl chain of octylamine by two carbons, to hexylamine, has a further deleterious effect on transfer; k_{cat}/K_m is now down by some 700-fold. This profile of acceptor substrate reactivity indicates that both the length of the NSPD chain and its three amines are determinants for transfer efficiency. The recognition of the distal, free amine might be important for preventing bisacylation of NSPD or 1,7-DAH; bisacylation was not observed for either substrate.

An examination of holo-VibB (Figure 8, Table 2) as a substrate for VibH shows a low K_m of 0.8 μM and a robust k_{cat} of 600 min^{-1}. Holo-EntB, covalently acylated with DHB by EntE, was also able to serve as a substrate from VibH, with catalytic efficiency down approximately 20-fold with respect to VibB. Thus, the DHB-*S*-pantetheine thioester requires the correct carrier protein domain for optimal acyl transfer; protein−protein interactions between the donor ArCP and VibH influence the acylation reaction.

The amide synthase activity demonstrated here for VibH is functionally similar to that performed by condensation domains of NRPS. The C domains are the central catalytic domains of an NRPS module and transfer an upstream, carrier-protein-linked thioester to a downstream nucleophile, usually the free amine of a carrier-protein-linked amino acid monomer. This condensation/translocation of the peptide chain determines the linear, directional growth of the product. C domains have been difficult to assay directly because they are embedded within larger NRPS proteins. As such both their substrates (linked to adjacent carrier proteins) and products are located in cis and in stoichiometric amounts, precluding the varying of concentration necessary for saturation kinetics and the turnover required to generate detectable amounts of product. Testing the substrate specificity of C domains has been difficult for similar reasons, although methods involving PPTase-mediated loading of aminoacyl-CoA analogues to bypass A domain specificity (*34*), as well as exploiting alternate substrates recognized by A domains (*23*), have offered information about C domain specificity. The approach of removing C domains from their NRPS contexts and expressing them as individual constructs has been slowed by lack of knowledge about domain boundaries and the uncertainty of domain stability. It is only recently that a C domain has been assayed directly: the enterobactin EntF C domain has been shown to condense the soluble downstream acceptor analogue seryl−*S*−(*N*-acetylcysteamine) with the natural upstream donor DHB−*S*−EntB to form DHB-serine (*35*).

VibH represents a novel amide synthase homologous to these C domains that is, by contrast, readily assayable. Of the seven conserved motifs of C domains (*4*), C1−C4 are at least partially present in VibH, as is C6. More importantly, the core catalytic motif, HHxxxDGxS, is present as HHIV-LDGYG. Mutational analysis of the second His and the Asp have proven them critical for condensation function (*31*).

Unlike standard C domains, however, VibH does not interact with two carrier protein domains but rather with only one, an upstream ArCP (VibB). This upstream, in trans interaction is precedented in the EntB/EntF pair and in the starter units of several other aryl-capped siderophores (*36*). The downstream substrate, the soluble, non-protein-bound triamine norspermidine, is different from those in standard NRPSs in two ways: this substrate is not in cis with the C domain, and this substrate is not tethered through a thioester to a PCP domain. In this respect, VibH's activity is more like acyl transferases such as the histone acetyltransferase Hat1, which transfers an Ac-CoA-derived acetyl group to a lysine side-chain amine (*37*). There are also examples of PKS chain termination via amide or ester bond formation by discrete enzymes in the rifamycin PKS [RifF, a lactam-forming amide synthase (*38*)] and the lovastatin PKS [LovD, a transesterase (*39*)]. However, neither of these enzymes appears to be related to NRPS C or TE domains. VibH is the first free-standing NRPS condensation protein to be assayed, and its kinetics compare favorably to those measured for the C domain of EntF, which possesses a k_{cat} of 230 min^{-1} and K_ms of 7 mM for the unnatural substrate seryl-SNAC and 0.73 μM for EntB (*35*).

The substrates of VibH, one carrier-protein-bound and the other a small molecule, are consonant with the structure of the final product. Vibriobactin is an unusual NRPS product in that it is not recognizably a peptide oligomer, with defined N- and C-termini and a C-terminal carboxylate to provide a point of attachment to the NRPS's most downstream carrier protein domain. Instead, vibriobactin is a triacylated triamine, which implies that while the donor substrates can be (and are) carrier protein thioesters, the acceptor must be a soluble, small molecule amine (used three times), which is "untetherable" by the standard C-terminal thioester strategy. In addition, vibriobactin is asymmetrically acylated, with one primary and the secondary amine capped with 2-(2,3-dihydroxyphenyl)-5-methyloxazolinyl moieties and the remaining primary amine with 2,3-DHB. This requires that the acylating enzymes recognize and distinguish between the primary and secondary amines, as well as acylate only one primary amine on each molecule of norspermidine. VibH clearly demonstrates this ability here, which also indicates that its DHB acylation of norspermidine may be the first step of vibriobactin biosynthesis, as the remaining cappings can now occur exhaustively without regard to regiochemistry.

VibH's product, DHB-NSPD **2**, possesses one-third of the amine acylation activities necessary to produce vibriobactin. As this product is also a soluble small molecule, two further nonstandard NRPS condensation events are required to complete biosynthesis. In the accompanying paper (*40*), we present characterization of VibF, a multidomain NRPS subunit that encompasses all the remaining enzymatic activities for vibriobactin biosynthesis.

ACKNOWLEDGMENT

We are grateful to Elizabeth E. Wyckoff (Department of Microbiology, University of Texas, Austin) for sharing unpublished results and to Joan R. Butterton (Infectious Disease Division, Massachusetts General Hospital) for providing us with *V. cholerae* O395 genomic DNA. Preliminary sequence data for *V. cholerae* O1, strain N16961, was obtained from The Institute for Genomic Research website at http://www.tigr.org.

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BI001651A