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VibD and VibH Are Required for Late Steps in Vibriobactin Biosynthesis in *Vibrio cholerae*

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Vibrio cholerae **synthesizes the catechol siderophore vibriobactin. In this report, we present the complete map of a vibriobactin gene region containing two previously unreported vibriobactin biosynthetic genes. *vibD* encodes a phosphopantetheinyl transferase, and *vibH* encodes a novel nonribosomal peptide synthase. Both VibD and VibH are required for vibriobactin biosynthesis.**

Vibrio cholerae, like most other bacterial pathogens, requires iron for growth and survival, and it possesses multiple systems for iron acquisition (11, 14, 17, 20). One mechanism by which *V. cholerae* acquires iron is the synthesis and transport of the catechol siderophore vibriobactin (11). Vibriobactin is synthesized and secreted into the environment, where it binds ferric iron with high affinity. The ferri-vibriobactin complex is then transported into the cell by a process that requires the outer membrane receptor ViuA (6, 27), a functional TonB system (17), and an inner membrane permease system (31). The ViuB protein then removes the iron from the ferri-siderophore complex (4).

Vibriobactin contains three molecules of 2,3-dihydroxybenzoic acid (DHBA) linked either directly or through threonine residues to the polyamine norspermidine (Fig. 1) (11). Norspermidine is rarely synthesized by bacteria, but it is a common polyamine in members of the family *Vibrionaceae* (32, 33). Although the structure of vibriobactin is unique, its biosynthesis shares features with the synthesis of the prototype catechol siderophore enterobactin. The first committed steps in the synthesis of enterobactin lead to the synthesis of DHBA from chorismate (8, 29). Vibriobactin biosynthesis also requires the synthesis of DHBA from chorismate (11), and the pathway for DHBA synthesis appears to be the same in *V. cholerae* and in *Escherichia coli*. *V. cholerae* contains genes homologous to *entA*, *entB*, and *entC*, the three genes required for DHBA synthesis in *E. coli* (8, 29), and each of these genes in *V. cholerae*, *vibABC*, complements a defect in the homologous *ent* gene (30). In addition, a *V. cholerae vibA* mutant produces no DHBA, confirming that the role of these genes in *V. cholerae* is DHBA synthesis (30).

The mechanism of vibriobactin biosynthesis from DHBA, threonine, and norspermidine is different from the mechanism of enterobactin synthesis from DHBA and serine, but some aspects of the synthesis are conserved. In a recently proposed model of enterobactin biosynthesis (9), EntD is the phosphopantetheinyl transferase (15) which catalyzes the transfer of 4′-phosphopantetheine (pPant) to the side chain hydroxyl of a conserved serine residue within EntB. This posttranslational modification allows EntB to serve as the acyl carrier protein for DHBA. EntE catalyzes the adenylation of DHBA and transfer of the activated DHBA to the pPant moiety on EntB (10). EntF is a 142-kDa protein with four distinct domains (Fig. 2). The peptide carrier domain of EntF is covalently modified by the addition of a pPant moiety that allows it to act as the carrier protein for the serine moiety. This modification is catalyzed by EntD. All subsequent enzymatic reactions are catalyzed by EntF, including adenylation of serine and transfer of the activated serine to the endogenous pPant moiety (adenylation domain), formation of the amide bonds joining three DHBA molecules with three serines (condensation domain), and formation of the ester bonds which join the three serine-DHBA moieties to form the cyclic enterobactin molecule (thioester domain) (10, 22).

In vibriobactin biosynthesis, the mechanism to form the amide bonds that join the DHBA molecules to threonine or norspermidine and the threonine to the norspermidine backbone could be similar to the mechanism for transferring DHBA to serine in enterobactin. Genetic evidence also suggests that the mechanism of the late steps of vibriobactin and enterobactin biosynthesis may be similar, in that *V. cholerae* has homologues of *entBDEF*, the genes required for late steps in enterobactin biosynthesis (5, 30).

Unlike enterobactin, for which the biosynthetic and transport genes are located in a single 22-kbp genetic locus (8), vibriobactin genes are located in two separate genetic clusters (4–6, 27, 30, 31). Both gene clusters are located on *V. cholerae* replicon 1 but are separated by approximately 10^6 bp (13, 28). Each cluster contains both biosynthetic genes and genes for vibriobactin utilization. One of these clusters contains the vibriobactin transport and utilization genes *viuA* and *viuB* (4, 6) and the biosynthetic gene *vibF* (5). The second region (Fig. 3), which is the subject of this report, includes the previously described genes for the synthesis of DHBA from chorismate (*vibABC*) and a gene for the activation of DHBA (*vibE*) (30). The region also contains genes for a periplasmic binding protein-dependent ABC transport system, which transports vibriobactin and enterobactin through the periplasm and across the inner membrane.

Vibriobactin gene cluster contains an *entD* homologue that is required for vibriobactin biosynthesis. To identify all of the

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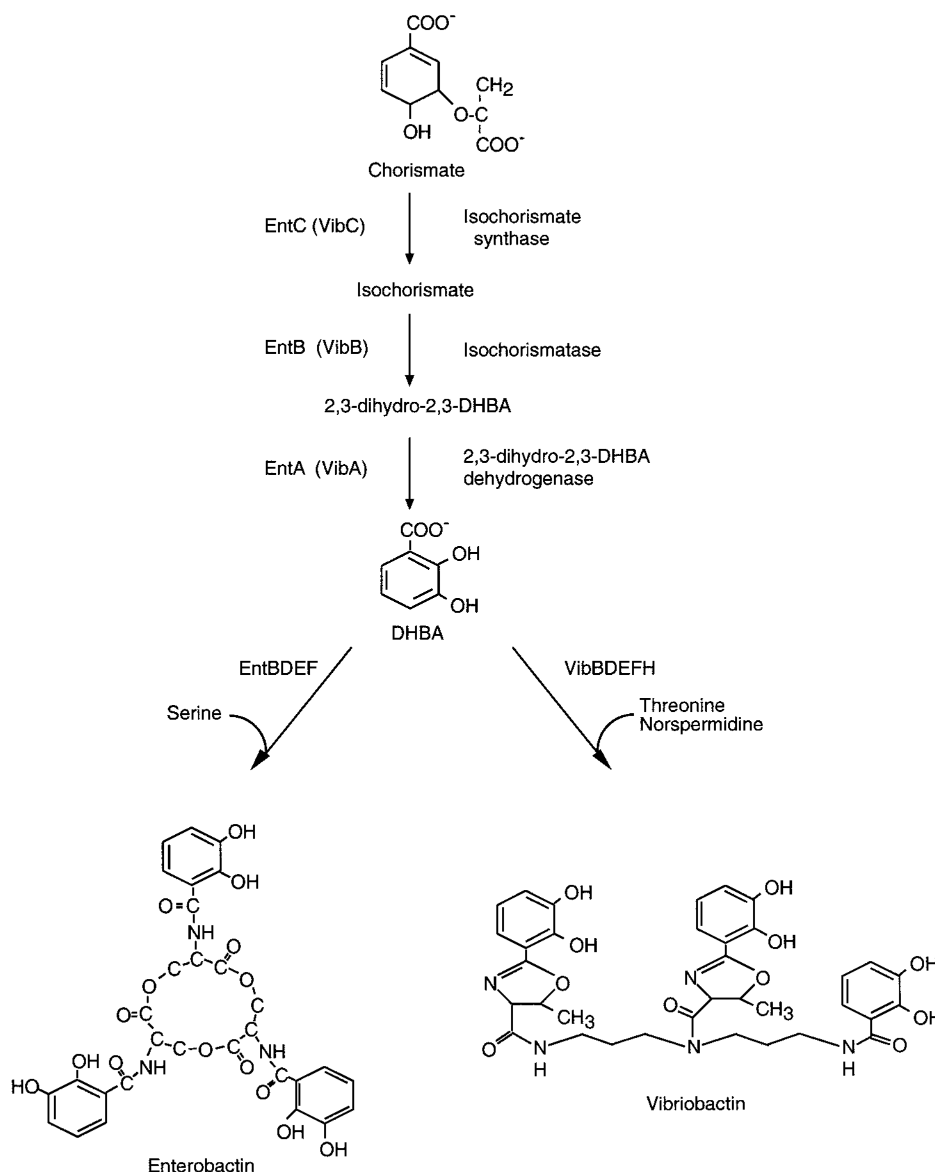

FIG. 1. Biosynthetic pathways and structures of the catechol siderophores enterobactin and vibriobactin (11).

genes required for vibriobactin biosynthesis and transport, the DNA sequence of the entire vibriobactin region was determined. Two genes, *vibD* and *vibH*, that have not been described previously were identified, and their locations relative to the other vibriobactin genes are shown in Fig. 3.

One of these genes, *vibD*, encodes a protein with sequence homology to *E. coli* EntD (2) and other phosphopantetheinyl transferase proteins (15). A ClustalW alignment (18) of VibD and EntD sequences shows 31% amino acid identity and 16% conservative substitutions. VibD also contains regions with sequence similarity to each of the two phosphopantetheinyl transferase superfamily consensus motifs (15) (Fig. 2). The assignment of *vibD* as the *V. cholerae entD* homologue is supported by the observation that *vibD* complements an *E. coli entD* mutation (33 and data not shown).

To determine whether *vibD* was required for vibriobactin biosynthesis, a *vibD* mutant, EWV101, was constructed by marker exchange as previously described (30). This strain and the other bacterial strains and plasmids used in this study are described in Table 1. EWV101 was positive for the synthesis of catechols (Table 2), indicating that the mutant had no defect in DHBA biosynthesis. To determine whether EWV101 could synthesize vibriobactin, the ability of EWV101 to cross-feed *V. cholerae vibD*, *vibB*, *vibH*, and *vibA* mutants was determined (Table 2). The *vibD* mutant failed to stimulate the growth of the *vibD* and *vibH* mutant strains, indicating that it was not secreting vibriobactin. The vibriobactin synthesis defect in this strain was complemented by either *vibD* or *entD* encoded on a plasmid (Table 2 and data not shown). Taken together, these data indicate that VibD is likely to provide the phosphopantetheinyl transferase activity required for vibriobactin synthesis. Sequences similar to the phosphopantetheinylation consensus sequence (9) are found in the potential target proteins VibB (FLGLDSI, amino acids 243 to 249) (30) and VibF

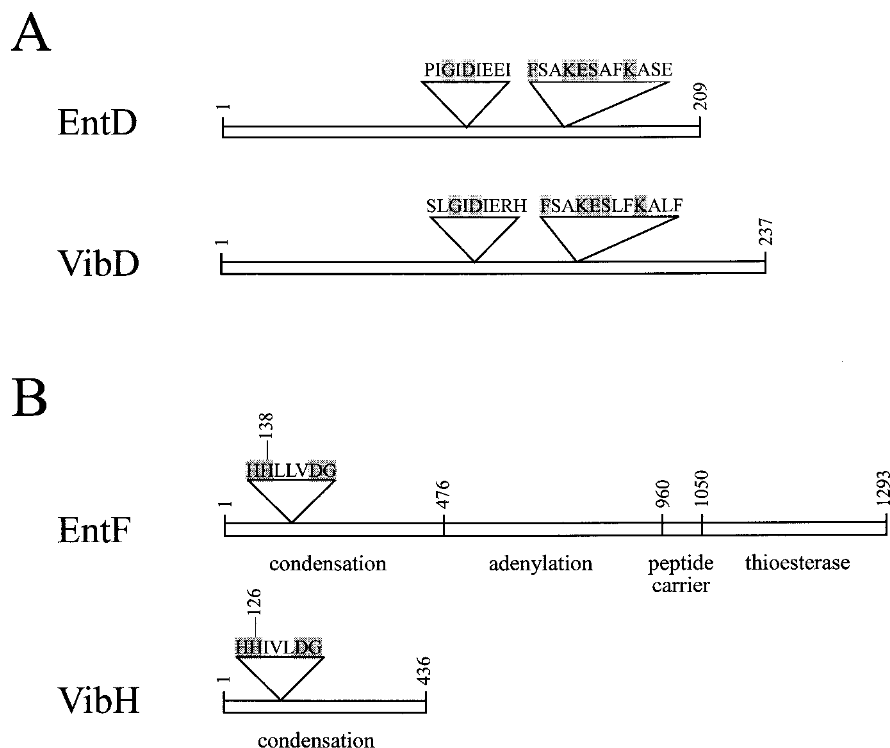

FIG. 2. (A) Schematic representation of EntD and VibD. The proposed phosphopantetheinyl transferase consensus regions are shown (15). The most highly conserved amino acid residues are shaded. (B) Domain structure of EntF and VibH. The EntF protein contains condensation (amino acids 1 to 475), adenylation (amino acids 476 to 960), peptide carrier (961 to 1049), and thioesterase (amino acids 1050 to 1293) domains (10, 22), while VibH contains only a condensation domain. The proposed catalytic region for the condensation domain is shown, and the most highly conserved amino acids are shaded. The approximate amino acid number at the junctions of the domains is indicated.

(DFGGHSL, amino acids 1886 to 1892) (5). The underlined serine residue within these sequences is the likely site of pPant addition by VibD.

EWV101 stimulated the growth of the *vibA* mutant (Table 2). This is likely due to secretion of DHBA by this *vibD* mutant. DHBA could be taken up and converted to vibriobactin by the *vibA* mutant, which has no defect in the genes required to convert DHBA to vibriobactin. In contrast, the *vibD* mutant did not stimulate growth of the *vibB* mutant EWV104 (Table 2). A role for VibB in the late steps of vibriobactin synthesis suggests that VibB, like its *E. coli* homologue EntB, is bifunctional, with the amino-terminal region of the protein containing the isochorismatase activity required for the synthesis of DHBA (30), while the carboxy-terminal region functions as the carrier protein for DHBA (9). This is supported by the observation that the VibB amino acid sequence contains the carrier protein consensus sequence FLGLDSI at amino acids 243 to 249 (9).

VibH is required for vibriobactin biosynthesis. An additional open reading frame was located between *vibA* and *viuP* (Fig. 3). To determine whether this gene, named *vibH*, was required for vibriobactin biosynthesis, a *vibH* mutant of *V. cholerae* was constructed. Like the *vibD* mutant, the *vibH* mutant strain, SSV119, was positive for the synthesis of catechols (Table 2), indicating that the conversion of chorismate to DHBA was not impaired. SSV119 did not cross-feed the *vibD*, *vibB*, or *vibH* mutants, indicating a defect in vibriobactin biosynthesis (Table 2). Providing the *vibH* gene in *trans* on plasmid pJSV78 restored the ability to stimulate growth of each of the mutant strains (Table 2). SSV119 did stimulate the growth of the *vibA* mutant, consistent with the ability of SSV119 to produce the catechol DHBA, as discussed above. Thus, the phenotype of the *vibH* mutant suggests that VibH, like VibD, is required for the assembly of vibriobactin from DHBA, threonine, and norspermidine.

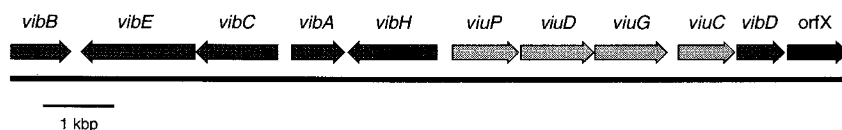

FIG. 3. Organization of the second vibriobactin gene region. The arrows indicate the direction of transcription of the vibriobactin genes. Vibriobactin biosynthetic genes are indicated with dark arrows, and the transport genes are shown with light gray arrows. OrfX is closely linked to *vibD*, but a role in vibriobactin transport or utilization has not been shown. The DNA sequence of this region is posted at GenBank accession number U52150.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
Lou15	*V. cholerae* E1 Tor	24
EWV100	*V. cholerae* Lou15 *vibA*::Tn5	30
EWV101	*V. cholerae* Lou15 *vibD*::mini-Tn*10kan*[a]	This study
EWV104	*V. cholerae* Lou15 *vibB*::*cam*[b]	This study
SSV119	*V. cholerae* Lou15 *vibH*::*cam*[c]	This study
DH5α	*E. coli* host for subcloning	12
Plasmids		
pBluescript SK−	Cloning vector	Strategene
pJSV78	7.8-kbp *Sal*I fragment with *vibCAH*	30
pJSV90	9.0-kbp *Eco*RI fragment with *vibH*, *viuPDGC*, and *vibD* in pACYC184	30
pVIB115	1.9-kbp *Hin*dIII-*Stu*I fragment with *vibD* in pBluescript SK−	This study

[a] The site of the mini-Tn*10kan* insertion in *vibD* is indicated in GenBank accession number U52150.
[b] The *cam*cassette is inserted into the *Nsi*I site in *vibB*.
[c] The *cam*cassette is inserted into the *Bgl*II site in *vibH*.

Analysis of predicted VibH protein sequence. The predicted VibH protein has a calculated molecular mass of 49.8 kDa and a predicted pI of 5.8. A Blast search (1) revealed that VibH has sequence homology with nonribosomal peptide synthase proteins, including *Bacillus subtilis* DhbF (GenBank accession no. Z99120) (21), *Streptomyces coelicolor* A3(2) calcium-dependent antibiotic synthase I (GenBank AL035640) (19), *E. coli* EntF (22), *Streptomyces chrysomallus* actinomycin synthetase II (23), and *Serratia liquefaciens* SwrA (16). These homologies suggest that VibH is a member of the nonribosomal peptide synthase family. However, VibH is much smaller than other nonribosomal peptide synthase proteins. This makes VibH an atypical member of a family in which the proteins generally have molecular weights of greater than 100,000. The unusually small predicted size of VibH cannot be explained by a frameshift or other sequencing error, since *vibA* is located immediately downstream of the *vibH* termination codon (Fig. 3).

Alignment of VibH with the best characterized of the closely related proteins, EntF, revealed that VibH protein aligns well with the first 452 amino acids of EntF. A ClustalW alignment shows 24% amino acid identity and 17% conservative amino acid substitutions. This region of EntF is the condensation domain of the protein, suggesting that VibH has a condensation function. This is supported by the observation that the sequence HHIVLDG (VibH amino acids 125 to 131) matches the condensation domain consensus sequence HHXXXDG (7, 26). The second histidine of this sequence is the catalytic residue. An aligned map of the VibH and EntF domain structures is shown in Fig. 2.

TABLE 2. Effect of *vibD* and *vibH* mutations on catechol and vibriobactin synthesis[a]

Indicator strain (genotype)	Catechol synthesis[b]	Zone of stimulation (mm) with:				
		Lou15 (wild type)	EWV101	EWV101 (pVIB115)	SSV119	SSV119 (pJSV78)
EWV101 (*vibD*)	+	30	0	16	0	24
EWV104 (*vibB*)	−	31	0	13	0	18
SSV119 (*vibH*)	+	31	0	15	0	23
EWV100 (*vibA*)	−	33	22	25	22	25

[a] Cultures of the indicator strains were seeded into L agar containing EDDA (100 μg/ml). The indicated *V. cholerae* strains were spotted onto the medium. The zone of growth was measured 18 h after inoculation. pVIB115 encodes *vibD*, and pJSV78 encodes *vibH*.
[b] Catechol synthesis was determined by the method of Arnow (3).

Nonribosomal peptide synthases have a modular structure in which a condensation domain is present together with an adenylation domain and a peptide carrier domain, which is the site of pPant attachment. VibH contains only the condensation domain, and no regions of homology to either an adenylation domain (25) or a peptide carrier domain (9) are present (Fig. 2). This unusual protein structure raises questions about the mechanism of action of VibH. Usually the substrate of a condensation domain is the amino acid attached to the pPant moiety of the peptide carrier domain. Since it is not expected that VibH would contain such a covalently attached amino acid, it is unclear how this protein identifies its substrates. The observation that VibH has only one of the domains present in EntF is consistent with our previous observation that plasmid pJSV90, which contains *vibH*, does not complement an *E. coli entF* mutation (30).

Conclusions. Genomic data indicate that all of the genes for vibriobactin synthesis have now been identified (13). At least four distinct coupling reactions must occur during the assembly of vibriobactin from DHBA, threonine, and norspermidine (Fig. 1). One molecule of DHBA is joined directly with a primary amine on norspermidine, the other two DHBA molecules are joined to the cyclized threonines, and the threonine-DHBA conjugates are joined to the norspermidine at either a primary or a secondary amine. It is not known which of these reactions is catalyzed by VibH. The other nonribosomal peptide synthase homologue required for vibriobactin biosynthesis, VibF (5), is a very large protein (269.5 kDa). It is believed that VibF catalyzes all the late steps not performed by VibH that are required for assembly of the vibriobactin molecule.

This work completes the identification of the vibriobactin biosynthesis genes in *V. cholerae.* It is unclear why the genes for vibriobactin synthesis and transport are divided into two genetic loci, but the separation of genes that usually map together has been observed for other iron acquisition systems in *V. cholerae.* For example, the heme receptor gene *hutA* maps at a distance from the other heme transport genes (13, 28). Both of the vibriobactin regions map to chromosome 1, which contains most of the genes required for growth and pathogenicity of *V. cholerae* (13). This may reflect the central role of vibriobactin synthesis and utilization in the growth and survival of *V. cholerae* in at least one of its habitats.

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