

Vibrio cholerae VibF Is Required for Vibriobactin Synthesis and Is a Member of the Family of Nonribosomal Peptide Synthetases

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A 7.5-kbp fragment of chromosomal DNA downstream of the *Vibrio cholerae* vibriobactin outer membrane receptor, *viuA*, and the vibriobactin utilization gene, *viuB*, was recovered from a *Sau3A* lambda library of O395 chromosomal DNA. By analogy with the genetic organization of the *Escherichia coli* enterobactin gene cluster, in which the enterobactin biosynthetic and transport genes lie adjacent to the enterobactin outer membrane receptor, *fepA*, and the utilization gene, *fes*, the cloned DNA was examined for the ability to restore siderophore synthesis to *E. coli* *ent* mutants. Cross-feeding studies demonstrated that an *E. coli* *entF* mutant complemented with the cloned DNA regained the ability to synthesize enterobactin and to grow in low-iron medium. Sequence analysis of the cloned chromosomal DNA revealed an open reading frame downstream of *viuB* which encoded a deduced protein of greater than 2,158 amino acids, homologous to *Yersinia* sp. HMWP2, *Vibrio anguillarum* AngR, and *E. coli* EntF. A mutant with an in-frame deletion of this gene, named *vibF*, was created with classical *V. cholerae* strain O395 by in vivo marker exchange. In cross-feeding studies, this mutant was unable to synthesize ferric vibriobactin but was able to utilize exogenous siderophore. Complementation of the mutant with a cloned *vibF* fragment restored vibriobactin synthesis to normal. The expression of the *vibF* promoter was found to be negatively regulated by iron at the transcriptional level, under the control of the *V. cholerae* *fur* gene. Expression of *vibF* was not autoregulatory and neither affected nor was affected by the expression of *irgA* or *viuA*. The promoter of *vibF* was located by primer extension and was found to contain a dyad symmetric nucleotide sequence highly homologous to the *E. coli* Fur binding consensus sequence. A footprint of purified *V. cholerae* Fur on the *vibF* promoter, overlapping the Fur binding consensus sequence, was observed using DNase I footprinting. The protein product of *vibF* is homologous to the multifunctional nonribosomal protein synthetases and is necessary for the biosynthesis of vibriobactin.

Iron is a required element for bacterial survival and growth. Under aerobic conditions, however, iron is present only in insoluble mineral complexes or bound to a mammalian host's own iron-binding proteins (11, 12). *Vibrio cholerae*, like many pathogenic bacteria, produces a siderophore—a low-molecular-weight iron chelator—which effectively competes with human iron-binding proteins, such as lactoferrin and transferrin (12, 35). The *V. cholerae* siderophore vibriobactin belongs to the group of catechol siderophores, of which enterobactin, a cyclic trimer of 2,3-dihydroxybenzoyl-L-serine produced by *Escherichia coli*, is the prototype (35). Vibriobactin contains three residues of 2,3-dihydroxybenzoic acid (2,3-DHBA) and two residues of threonine, which are in the form of oxazoline rings (21). The polyamine backbone of vibriobactin is unusual in being the rare compound norspermidine [*N*-(-aminopropyl)-1,3-diaminopropane] rather than spermidine, which is found in the *Paracoccus denitrificans* siderophore parabactin and the *Agrobacterium tumefaciens* siderophore agrobactin (21). The biosynthetic steps needed for vibriobactin production are not yet clearly defined.

In *E. coli*, the enterobactin biosynthetic (*ent*) and transport (*fep*) genes lie in a large gene cluster, with the clockwise order of the enterobactin genes on the *E. coli* chromosome being

entD, *fepA* (which encodes the enterobactin outer membrane receptor), *fes* (which is involved in ferric enterobactin uptake and processing), *entF*, *fepE*, *fepC*, *fepG*, *fepD*, *fepB*, *entE*, *entB*, and *entA*. Similarly, the gene encoding the vibriobactin outer membrane receptor, *viuA*, lies upstream of a second gene, *viuB*, which encodes a cytoplasmic protein analogous in function to *fes* (5, 6). By analogy with the enterobactin gene cluster, other genes involved in vibriobactin biosynthesis and uptake may be located near *viuA* and *viuB*. However, recent work has identified a group of vibriobactin biosynthetic (*vib*) and utilization (*viu*) genes that are not linked to the *viuA*-*viuB* region (GenBank accession no. U52150) (52). These include genes postulated to function in the synthesis of 2,3-DHBA from chorismate (*vibC*, *vibB*, and *vibA*), a vibriobactin synthase cluster linking the three residues of 2,3-DHBA to two residues of threonine with the norspermidine backbone (*vibBDEH*), and several genes with transport function (*viuPDGC*). The order of these genes on the *V. cholerae* chromosome, *vibB*, *vibE*, *vibC*, *vibA*, *vibH*, *viuP*, *viuD*, *viuG*, *viuC*, and *vibD*, is quite different from that of the enterobactin gene cluster, and no congener of *entF* is present in this cluster.

The production of vibriobactin is negatively regulated by iron, and many of the genes involved in vibriobactin production and uptake have been found to be negatively regulated by iron at the transcriptional level, under the control of the iron repressor protein Fur. The transcription of both *viuA* and *viuB* is derepressed under high-iron conditions in a *fur* mutant (5, 6), while the uptake and utilization genes *vibB*, *vibC*, *vibH*, *viuP*, and *viuC* all have potential Fur binding consensus sequences in their putative promoter regions (GenBank acces-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype ^a	Reference or source
<i>V. cholerae</i> strains		
O395	Wild-type <i>V. cholerae</i> , classical biotype; Sm ^r	31
MBG14	O395 <i>viuA</i> ::Tn <i>phoA</i> ; Sm ^r Km ^r	6
MBG40	O395 <i>irgA</i> ::Tn <i>phoA</i> ; Sm ^r Km ^r	20
CML19	O395 Δ <i>fur</i> ; Sm ^r	30
JRB3	O395 Δ <i>irgB</i> ; Sm ^r	50
JRB15	O395 <i>lacZ</i> :: <i>vibFp</i> → <i>phoA</i> ; Sm ^r	This study
JRB16	O395 Δ <i>fur lacZ</i> :: <i>vibFp</i> → <i>phoA</i> ; Sm ^r	This study
JRB17	O395 Δ <i>irgB lacZ</i> :: <i>vibFp</i> → <i>phoA</i> ; Sm ^r	This study
JRB18	O395 Δ <i>vibF</i> ; Sm ^r	This study
JRB19	MBG14 Δ <i>vibF</i> ; Sm ^r Km ^r	This study
JRB20	MBG40 Δ <i>vibF</i> ; Sm ^r Km ^r	This study
JRB21	O395 Δ <i>vibF lacZ</i> :: <i>vibFp</i> → <i>phoA</i> ; Sm ^r	This study
JRB22	O395 <i>irgA lacZ</i> :: <i>vibFp</i> → <i>phoA</i> ; Cm ^r Sm ^r	This study
JRB23	O395 <i>viuA lacZ</i> :: <i>vibFp</i> → <i>phoA</i> ; Tc ^r Sm ^r	This study
<i>E. coli</i> strains		
DH5 α	F ⁻ <i>deoR endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1</i> Δ (<i>argF-lacZYA</i>)U169 (ϕ 80d <i>lacZ</i> Δ M15)	BRL ^b
AB1515	<i>purE42 proC14 leu-6 trpE38 thi-1 fhuA23 lacY1 mtl-1 xyl-5 rpsL109 azi-6 tsx-67</i>	S. Payne; 45
AN117	<i>entF</i> derivative of AB1515	52
SM10 λ pir	<i>thi thr leu tonA lacY supE recA</i> ::RP4-2-Tc::Mu λ pirR6K; Km ^r	34
SY327 λ pir	Δ (<i>lac-pro</i>) <i>nalA recA56 araD argE</i> (Am) λ pirR6K; Sm ^r	34
Plasmids		
pGEM-T	PCR cloning vector; Amp ^r	Promega Corp.
pCVD442	Suicide vector composed of <i>mob</i> , <i>ori</i> , and <i>bla</i> regions of pGP704 and <i>sacB</i> gene of <i>B. subtilis</i> ; Amp ^r	16
pBluescript II SK+	Cloning vector; Amp ^r	Stratagene
pUJ10	pUC18-based plasmid with multiple cloning site between divergent <i>E. coli lacZ</i> and <i>phoA</i> genes; Amp ^r	15
p6891MCS	pBR327 containing 8-kbp <i>Sau3A</i> fragment of <i>V. cholerae</i> DNA encoding <i>lacZ</i> and multiple cloning site inserted at <i>KpnI</i> within <i>lacZ</i> ; Amp ^r	4
pJRB20	pBluescript II SK+ with 7.5-kbp cloned <i>SacI</i> insert from <i>Sau3A</i> LambdaGEM-11 library of <i>V. cholerae</i> chromosomal DNA, containing 5' end of <i>vibF</i> ; Amp ^r	This study
pMHC4	pGEM-T with 760-bp cloned insert of <i>V. cholerae</i> chromosomal DNA downstream of region cloned into pJRB20; Amp ^r	This study
pWCW1	pBluescript II SK+ with cloned 1,552-bp <i>HindIII</i> fragment within <i>vibF</i> from pJRB20; Amp ^r	This study
pWCW2	pWCW1 with 882-bp in-frame deletion of <i>vibF</i> between two internal <i>BglII</i> sites; Amp ^r	This study
pWCW3	pCVD442 with <i>SalI-SacI</i> fragment from pWCW2 containing 882-bp in-frame deletion of <i>vibF</i> ; Amp ^r	This study
pMHC1	pUJ10 with 228-bp <i>XbaI-BamHI</i> fragment containing intergenic region of <i>viuB-vibF</i> , such that <i>vibFp</i> → <i>phoA</i> ; Amp ^r	This study
pJRB44	p6891MCS with 2.8-kbp <i>NotI</i> fragment from pMHC1 containing <i>vibFp</i> → <i>phoA</i> ; Amp ^r	This study
pPAC19	pCVD442 with 3.7-kbp <i>SacI-SphI</i> fragment containing <i>irgA</i> ::Cm ^r ; Amp ^r Cm ^r	48
pPAC20	pCVD442 with 4.2-kbp <i>PvuII</i> fragment containing <i>viuA</i> ::Tc ^r ; Amp ^r Tc ^r	48

^a Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance, Tc^r, tetracycline resistance.

^b BRL, Bethesda Research Laboratories Life Technologies, Inc.

sion no. U52150) (52). In addition to the production of vibriobactin, *V. cholerae* possesses other iron uptake systems, which use the iron in ferric citrate, ferrichrome, hemin, or hemoglobin (44, 47). The heme transport system also depends on a gene cluster that is controlled by Fur (24, 25, 36). The gene encoding the 70-kDa major iron-regulated outer membrane protein of *V. cholerae*, IrgA, is negatively controlled by Fur but is also under the positive transcriptional regulation of the product of a divergently transcribed gene, *irgB* (17–20, 50). IrgA is a virulence factor in *V. cholerae* but has not yet been demonstrated to play a role in iron acquisition. The interplay of transcriptional regulators in the control of iron-regulated genes in *V. cholerae* remains to be investigated.

In this study, we have characterized an additional gene in the vibriobactin biosynthetic and uptake apparatus. The protein product of this gene is a congener of EntF and is homologous to a family of proteins involved in the biosynthesis of siderophores and peptide antibiotics. We describe the phenotype

of a deletion mutant form of this gene, examine its regulation by iron and Fur and its interaction with other iron-regulated genes, and present studies that suggest that the protein product of this gene acts as a multifunctional nonribosomal peptide synthetase in the biosynthesis of vibriobactin.

(Portions of this work were presented at the 97th General Meeting of the American Society for Microbiology [abstr. B-235].)

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1.

Media. All strains were preserved by storage at -70°C in Luria broth (LB) medium (43) containing 15% glycerol. LB medium, with or without the addition of the iron chelator 2,2-dipyridyl (final concentration, 0.2 mM), was used for growth under low- and high-iron conditions, respectively. Ampicillin (100 $\mu\text{g/ml}$), chloramphenicol (12.5 $\mu\text{g/ml}$), tetracycline (2 $\mu\text{g/ml}$), and streptomycin (100 $\mu\text{g/ml}$) were added as appropriate.

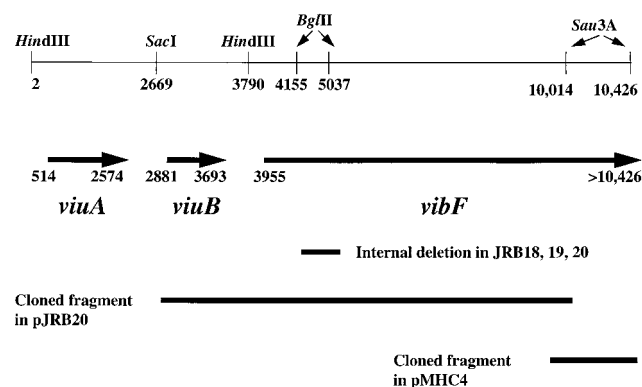


FIG. 1. Partial restriction map of O395 chromosomal DNA with relevant restriction enzyme sites, locations of the cloned fragments in pJRB20 and pMHC4, and position of the internal deletion of *vibF* in JRB18, JRB19, and JRB20. The extents of the coding regions of *viiA*, *viiB*, and *vibF* are indicated by solid arrows. The numbering of sites corresponds to that in reference 5.

Genetic methods. Isolation of plasmid DNA, preparation of RNA, restriction enzyme digests, agarose gel electrophoresis, plaque hybridization, and Southern hybridization of DNA separated by electrophoresis were performed by standard molecular biology techniques (43). Bacterial chromosomal DNA was prepared using the Easy-DNA Kit (Invitrogen Corp., Carlsbad, Calif.). Genescreen Plus and Colony/PlaqueScreen hybridization transfer membranes (NEN Research Products, Boston, Mass.) were used in accordance with the manufacturer's protocols for Southern and plaque hybridizations. Primer extension analysis was performed as previously described (33); oligonucleotide primers were hybridized to RNA in 0.4 M NaCl–40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], pH 6.4, without formamide, at 55°C for 2 h. RNasin and avian myeloblastosis virus reverse transcriptase were obtained from Bethesda Research Laboratories Life Technologies, Inc. (Gaithersburg, Md.). DNA sequencing was performed at the Massachusetts General Hospital Department of Molecular Biology DNA Sequencing Core Facility using ABI Prism DiTerminator Cycle sequencing with AmpliTaq DNA polymerase FS and an ABI 377 DNA sequencer (Perkin-Elmer Applied Biosystems Division, Foster City, Calif.).

Plasmids were transformed into *E. coli* strains by standard heat shock techniques or electroporated into *V. cholerae* by using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) in accordance with the manufacturer's protocol, with modifications for electroporation into *V. cholerae* as previously described (19). *E. coli* strains DH5 α and SY327 λ pir were used as recipients for the plasmid constructs described below. DNA restriction endonucleases and T4 DNA ligase were used in accordance with the manufacturers' specifications. Restriction enzyme-digested chromosomal and plasmid DNA fragments were separated on 1% agarose gels; fragments of interest were cut from the gel under UV illumination and purified using GenElute spin columns (Supelco Inc., Bellefonte, Pa.). DNA fragments used as probes were radiolabeled with [α -³²P]dCTP using the Prime-It II random priming labeling kit (Stratagene, La Jolla, Calif.).

The PCR was carried out using a standard DNA minicycler (M. J. Research, Inc., Watertown, Mass.). All PCRs were performed using the following conditions: 94°C for 4 min; 30 cycles of 94°C for 1 min (denaturing), 50°C for 1 min (annealing), and 72°C for 1 min (extension); and 72°C for 5 min (terminal extension).

Construction of plasmids. A 7.5-kbp fragment of *V. cholerae* O395 chromosomal DNA downstream of the gene encoding the vibriobactin outer membrane receptor, *viiA*, and the vibriobactin utilization gene, *viiB*, was recovered from a *Sau3A* LambdaGEM-11 (Promega Corp., Madison, Wis.) library of O395 chromosomal DNA (gift of Ken Peterson) as follows. Plaques were hybridized with a 1,485-bp *SacI* (bp 2669)-to-*BglII* (bp 4155) DNA fragment probe encompassing *viiB* (5, 6) (Fig. 1). Bacteriophage DNA was isolated from hybridizing plaques by standard techniques (43). A 7.5-kbp *SacI* fragment containing O395 chromosomal DNA was isolated from one of the hybridizing bacteriophages and ligated into the unique *SacI* site of pBluescript II SK+ (Stratagene) to create plasmid pJRB20. One of the flanking *SacI* sites was from O395 chromosomal DNA; the other was from the LambdaGEM-11 vector, in which a *SacI* site is adjacent to the *BamHI* site within which the *Sau3A* chromosomal fragments were cloned.

Sequence analysis of the fragment in pJRB20 revealed a single long open reading frame (see below), which we called *vibF*. Chromosomal DNA downstream of the insert in pJRB20 was isolated by PCR amplification of the hybridizing bacteriophages with primers complementary to the multiple cloning site of the right arm of the LambdaGEM-11 cloning vector (5' CCATTTAGGTGAC ACTATGAA 3') and to the previously sequenced downstream region of *vibF* (5' AGTTTCCCTAATGCCAAG 3'). A 760-bp PCR product was amplified from one of the isolated hybridizing phages and cloned into PCR cloning vector pGEM-T (Promega) to create plasmid pMHC4 (Fig. 1). Both strands of the

chromosomal DNA insert in pMHC4 were sequenced. Primers complementary to internal sequences of the pMHC4 chromosomal insert were used to amplify fragments from O395 chromosomal DNA which were then resequenced to confirm that the sequence obtained from the bacteriophage library was identical to those in the *V. cholerae* chromosome (data not shown).

An in-frame deletion of *vibF* was constructed as follows. pJRB20 was digested with *HindIII*, and the 1,552-bp *HindIII* fragment within *vibF* was cloned within the unique *HindIII* site of pBluescript II SK+ to create pWCW1. An 882-bp in-frame deletion of *vibF* was made between the two *BglII* sites in pWCW1 to create pWCW2 (Fig. 1). pWCW2 was digested with *SacI* and *SacI*; the 670-bp fragment encompassing the *vibF* deletion was cloned into the *SacI* and *SacI* sites of plasmid pCVD442 to create pWCW3. pCVD442 is a suicide vector containing the *pir*-dependent R6K replicon, the ampicillin resistance determinant, and the *sacB* gene from *Bacillus subtilis* (16).

Construction of *V. cholerae vibF* mutants. The *V. cholerae vibF* mutant JRB18 was constructed as follows. pWCW3 was transformed into SM10 λ pir and then crossed with streptomycin-resistant *V. cholerae* O395. Streptomycin- and ampicillin-resistant merodiploids were subsequently grown overnight in LB medium without ampicillin selection, plated on LB agar containing 10% sucrose but no NaCl, and grown at 30°C for 30 h. Because the product of the *sacB* gene is lethal in the presence of sucrose, surviving colonies had deleted the integrated *sacB* gene via a second recombination event and lost the resulting plasmid (3). In particular, sucrose-resistant colonies that were ampicillin susceptible had either re-excised the original plasmid to yield the parent O395 or had resolved the merodiploid state to replace the *vibF* locus in O395 with the *vibF* deletion from pWCW3. These two possibilities were distinguished by colony blotting and Southern hybridization (data not shown). One of these colonies was purified and named JRB18. Using the above methods, *vibF* deletions were also made in *V. cholerae* strains MBG14 (O395 *viiA*::TnphoA) and MBG40 (O395 *irgA*::TnphoA) to create JRB19 and JRB20, respectively.

Construction of alkaline phosphatase reporter strains. Reporter strains JRB15, JRB16, and JRB17 were constructed from *V. cholerae* strains O395, CML19 (*Afur*), and JRB3 (*AirgB*) as follows. A 228-bp DNA fragment containing the intergenic region of *viiB-vibF* was amplified by PCR (oligonucleotides 5'-AAATCTAGAGCGCGCCATTCACCTTGCCGTGTA-3' and 5'-TTTGG ATCCTAAACCCACAGATTCATCCC-3') with flanking 5' *XbaI*-*NotI* and 3' *BamHI* restriction sites and inserted into the *XbaI* and *BamHI* sites within the multiple cloning site of pUJ10, a pUC18-based plasmid containing a multiple cloning site between divergent *E. coli lacZ* and *phoA* genes (15), such that the *vibF* promoter controls the transcription of *phoA*, to create pMHC1. After cloning, this insert was fully sequenced, including both junctions. A 2.8-kbp fragment containing the *viiB-vibF* intergenic region, as well as the flanking *phoA* reporter gene, was removed by *NotI* digestion and ligated into the unique *NotI* site of p6891MCS, a plasmid containing a multiple cloning site between two fragments of the *V. cholerae lacZ* gene (4), to create plasmid pJRB44. This construct was introduced by *in vivo* marker exchange into the *lacZ* genes of *V. cholerae* strains O395, CML19, and JRB3, as previously described (4), to create strains JRB15, JRB16, and JRB17, respectively. Correct insertion of the construct within *lacZ* was confirmed by Southern blot using the 2.1-kbp *HpaI* fragment of p6891MCS as a probe (data not shown).

Strains JRB21 (Δ *vibF*), JRB22 (*irgA*), and JRB23 (*viiA*) were constructed as follows. A *vibF* deletion was made in reporter strain JRB15 by *in vivo* marker exchange with pWCW3 as described above to create JRB21. *irgA* and *viiA* mutations were made in JRB15 by similar methods using previously described plasmids pPAC19 and pPAC20 (48) to create JRB22 and JRB23.

Bioassay. Utilization of vibriobactin was determined by bioassay (36). Organisms (10⁵/ml) of indicator bacterial strains were solidified in iron-depleted medium (LB agar with ethylenediamine di[*o*-hydroxyphenylacetic acid] [EDDA] at 75 μ g/ml for *V. cholerae* strains or at 500 μ g/ml for *E. coli* strains) deferrated by the method of Rogers [40]). The ability of these strains to use vibriobactin was determined by measuring the growth of the indicator strains around 10- μ l spots of stationary-phase bacterial cultures (producer strains) after incubation at 37°C for 24 to 48 h. The indicator strains would not grow in the absence of usable exogenous siderophore or iron. A solution of ferrous sulfate (18 mM) was used as a positive control.

Alkaline phosphatase reporter assay. *vibFp*→*phoA* reporter strains were grown overnight in LB with and without the iron chelator 2,2-dipyridyl. Overnight cultures were standardized for density of bacterial growth, pelleted, washed, and permeabilized as previously described (32). Measurement of the amount of hydrolysis of *p*-nitrophenyl phosphate (Amresco) by permeabilized cells allowed calculation of the alkaline phosphatase activity. Test samples were prepared in triplicate, and strain O395 lacking the reporter construct was used as a negative control to account for endogenous phosphatase activity.

DNase I footprinting assay. Various amounts of purified Fur (50, 51) were incubated with a biotin end-labeled 228-bp DNA fragment containing the intergenic region of *viiB-vibF* described above. The binding buffer contained 20 mM Tris (pH 7.5), 5 mM MgCl₂, 50 mM NaCl, bovine serum albumin at 100 μ g/ml, salmon sperm DNA at 5 μ g/ml, 100 μ l of MnCl₂, and 10% glycerol. One picomole of DNA and the designated amount of purified Fur were added to a 95- μ l sample volume and allowed to incubate at room temperature for 30 min. Samples were subsequently incubated with bovine pancreatic DNase I (Worthington Biochemical Corporation, Freehold, N.J.) for exactly 30 s. The reaction

was stopped by addition of 10 μ l of a solution of 0.125 M EDTA and tRNA at 2.5 mg/ml, and DNA fragments were precipitated with ethanol. Samples were resuspended in a loading buffer consisting of 8 M urea, 40 mM Tris (pH 8.45), 40 mM borate, 0.05% bromophenol blue, and 0.05% xylene cyanol and stored at -80°C until use. A Maxam-and-Gilbert G + A reaction was performed on the same DNA fragment used for footprinting, and this was used as a position marker (30a). Samples were run on a 5% polyacrylamide gel, transferred to a Sequenase Images nylon membrane (United States Biochemical Corp.) by contact blotting, and developed using the Sequenase Images Detection Kit.

DNA and protein database searches. Nucleotide and derived amino acid sequences were assembled and initially analyzed using DNA Strider software version 1.0 (Christian Marck, Commissariat a l'Energie Atomique, Paris, France). Database comparisons were performed with the Basic Local Alignment Search Tool programs (1) using the server at the National Center for Biotechnology Information. Preliminary sequence data was obtained from the website of The Institute for Genomic Research (<http://www.tigr.org>). Protein homology searches were performed using the FASTA program, version 3.26, using the server at the European Bioinformatics Institute (37). A homology comparison to a database of conserved protein motifs was performed with the BLOCKS program (26). The hydropathicity index profile was calculated by using the Kyte-Doolittle formula (28).

Nucleotide sequence accession number. The GenBank accession number for the *vibF* sequence presented here is AF030977.

RESULTS

Complementation of an *E. coli entF* mutant with the *V. cholerae* chromosomal insert in pJRB20. *V. cholerae* chromosomal DNA downstream of the gene encoding the vibriobactin outer membrane receptor, *viuA*, and the utilization gene, *viuB*, was recovered as a 7.5-kbp *SacI* insert in pJRB20. In *E. coli*, *entF*, which encodes a cytoplasmic protein required for enterobactin biosynthesis, is adjacent to *feoA*, encoding the enterobactin outer membrane receptor, and *fes*, which encodes a protein involved in utilization of ferric enterobactin. To investigate whether the cloned *V. cholerae* chromosomal DNA might have EntF-like activity, the ability of pJRB20 to complement an *E. coli entF* mutant, AN117, was investigated. Wild-type parent *E. coli* strain AB1515, when complemented with the cloning vector pBluescript II SK+, grew to an optical density at 600 nm (OD_{600}) of 0.598 ± 0.117 (mean \pm standard deviation of triplicate cultures) following overnight growth in LB medium with added 2,2-dipyridyl. The *entF* mutant AN117(pBluescript II SK+) grew poorly under iron stress (OD_{600} of 0.036 ± 0.003). pJRB20 partially restored the growth of AN117 in low-iron medium to the level of the parent strain (OD_{600} of 0.338 ± 0.002).

The ability of the cloned *V. cholerae* chromosomal DNA on pJRB20 to complement the mutation in AN117 was also evaluated with a bioassay. In this assay, indicator strains do not grow in the absence of usable exogenous siderophore or iron; when siderophore or iron is present, a visible zone of growth can be seen around the siderophore-producing strains or iron compounds. The indicator strain used was the *entF* deletion mutant AN117, which is unable to synthesize ferric enterobactin but utilizes the siderophore normally. The producer strains tested were wild-type *E. coli* strain AB1515, the *entF* deletion mutant AN117, and AN117 complemented with pJRB20. The indicator strain grew when cross-fed by wild-type producer strain AB1515 (zone of growth, 18 mm) but had no zone of growth in the presence of the *entF* deletion mutant. The complemented mutant AN117(pJRB20) regained the ability to cross-feed the indicator strain (zone of growth, 15 mm), demonstrating that complementation of the *entF* mutant with pJRB20 restored enterobactin synthesis to normal.

Nucleotide sequence of *vibF*. The 7.5-kbp *V. cholerae* chromosomal insert in pJRB20 and the overlapping 760-bp insert in pMHC4 were cloned and sequenced as described above. One large open reading frame, which was named *vibF*, was identified (Fig. 1), beginning at bp 3955 and extending beyond the

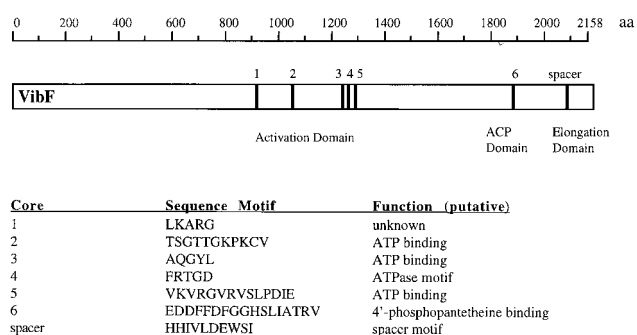


FIG. 2. Organization of domains and core sequence motifs within the peptide synthetase module identified from the deduced amino acid sequence of VibF. The relative locations of the core sequences (cores 1 to 6) and spacer motif, their amino acid sequences in one-letter code, and their putative functions are indicated. The locations of the activation, acyl carrier protein (ACP), and elongation domains are marked.

recovered chromosomal DNA, and therefore potentially encoding a deduced protein of greater than 2,158 amino acids. Three potential starting methionines were present, but a strongly conserved Shine-Dalgarno sequence was not identified.

Identification of components of a multifunctional nonribosomal peptide synthetase in VibF. The hydropathicity plot of VibF revealed no signal sequence or transmembrane domains (data not shown), suggesting that VibF is not a periplasmic or membrane protein but may be a cytoplasmic protein involved in ferric vibriobactin biosynthesis.

The FASTA algorithm for protein homology was used to compare VibF with other proteins in the SWISS-PROT database. VibF was highly homologous to a number of polypeptides involved in the biosynthesis of siderophores and peptide antibiotics, including *Yersinia* sp. HMWP2, a virulence factor involved in yersiniabactin synthesis (SWISS-PROT accession no. P48633; 24.9% identity in a 1,747-amino-acid overlap) (2, 22); *Vibrio anguillarum* AngR, a positive transcriptional regulator involved in anguibactin synthesis (accession no. P19828; 25.0% identity in a 984-amino-acid overlap) (9, 49); *E. coli* EntF, which is required for enterobactin synthesis (accession no. P11454; 24.0% identity in a 987-amino-acid overlap) (41); *Bacillus brevis* gramicidin S synthetases (accession no. P14688 and P14687); and *B. subtilis* surfactin synthetases (accession no. P27206, Q08787, and Q04747) (10), serine-activating enzyme (accession no. P45745), and peptide synthetases (accession no. P39846 and P39845).

Using the BLOCKS program to search for conserved domains, motifs suggestive of components of one module of a multifunctional nonribosomal peptide synthetase were detected in the deduced amino acid sequence of VibF (13, 29, 38, 46). The organization of these sequence motifs within the N terminus of VibF is shown in Fig. 2. An activation or AMP-binding domain, belonging to the family of adenylate-forming enzymes, extends from amino acid 922 to amino acid 1325 of the predicted protein and contains five core sequence motifs. A phosphopantetheinyl attachment site is present within the acyl carrier protein domain, with a conserved sequence beginning at amino acid 1882 containing the core motif GGHSI. A spacer motif begins at amino acid 2083, within the presumptive elongation domain.

Bioassay of ferric vibriobactin production and utilization. To further assess the role of VibF in vibriobactin synthesis, an internal deletion of *vibF* was made in classical *V. cholerae* strain O395; this mutant strain, JRB18, was then complemented with

TABLE 2. Alkaline phosphatase activities in high- and low-iron media

Strain	Genotype	Alkaline phosphatase activity (U/A ₆₀₀ of cells) ^a	
		High iron ^b	Low iron ^c
O395	Wild type	1 ± 0	1 ± 0
MBG14	<i>viuA::TnpHoA</i>	1 ± 0	26 ± 8
MBG40	<i>irgA::TnpHoA</i>	2 ± 0	572 ± 316
JRB15	<i>lacZ::vibFp→phoA</i>	2 ± 0	112 ± 7
JRB16	<i>lacZ::vibFp→phoA fur</i>	413 ± 8	151 ± 46
JRB17	<i>lacZ::vibFp→phoA irgB</i>	2 ± 0	47 ± 19
JRB18	O395 <i>vibF</i>	1 ± 0	2 ± 0
JRB19	MBG14 <i>vibF</i>	1 ± 0	23 ± 3
JRB20	MBG40 <i>vibF</i>	1 ± 0	832 ± 96
JRB21	<i>lacZ::vibFp→phoA vibF</i>	2 ± 0	138 ± 5
JRB22	<i>lacZ::vibFp→phoA irgA</i>	2 ± 0	159 ± 8
JRB23	<i>lacZ::vibFp→phoA viuA</i>	2 ± 0	117 ± 32

^a Units of alkaline phosphatase activity reflect the mean ± the standard deviation of triplicate samples (rounded to the nearest unit) and include a normalization factor for the density of culture growth. *V. cholerae* O395 and JRB18 were used as negative controls to account for endogenous phosphatase activity.

^b High iron, LB medium.

^c Low iron, LB medium with 0.2 mM 2,2-dipyridyl.

the *vibF* fragment on plasmid pJRB20. Analysis of the ability of JRB18 to synthesize or utilize exogenous ferric vibriobactin was evaluated in a bioassay. JRB18 was used as the indicator strain, while the parent O395, *vibF* mutant JRB18, and JRB18 complemented with pJRB20 were the producer strains. The indicator strain grew when cross-fed by O395 (zone of growth, 40 mm) but did not grow in the presence of mutant JRB18. Complementation of the *vibF* mutant with the portion of *vibF* cloned on pJRB20 restored growth of the indicator strain (zone of growth, 20 mm).

Regulation of the *vibF* promoter. To investigate the regulation of *vibF* transcription in more detail, chromosomal *vibFp-phoA* operon fusions were constructed. The role of iron in *vibF* expression was determined by measuring the alkaline phosphatase activity of reporter strain JRB15 (O395 *lacZ::vibFp→phoA*) after overnight growth in medium with or without the iron chelator 2,2-dipyridyl. Reporter strains JRB16 and JRB17 were used to test whether *vibF* expression was influenced by the regulatory proteins Fur and IrgB, while strains JRB21, -22, and -23 were used to evaluate the influence of VibF, IrgA, and ViuA on *vibF* expression. As shown in Table 2, *vibF* was negatively regulated by iron at the transcriptional level, under the control of the *V. cholerae fur* gene. In addition, *vibF* expression was reduced approximately twofold in an *irgB* mutant ($P < 0.02$ by Student's *t* test). However, *vibF* expression was not autoregulatory and neither affected nor was affected by the expression of *irgA* or *viuA*.

Localization of the transcriptional start site and promoter of *vibF* by primer extension. Primer extension analysis with RNA from strain O395 grown in low-iron medium was performed with a 21-mer synthetic oligonucleotide, 5' CAAATT CGGCGTAGAGATGCG 3', complementary to the DNA sequence located 76 bp downstream of the methionine start codon of *vibF*. As shown in Fig. 3, primer extension analysis revealed a potential transcriptional start site 65 bp upstream of the most proximal translational start site (marked with an asterisk in Fig. 4). Possible -10 and -35 boxes of the *vibF* promoter, homologous to the *E. coli* consensus sequences, are indicated in Fig. 4.

Homology of the *vibF* promoter region to the Fur binding consensus sequence. The postulated promoter region of *vibF*

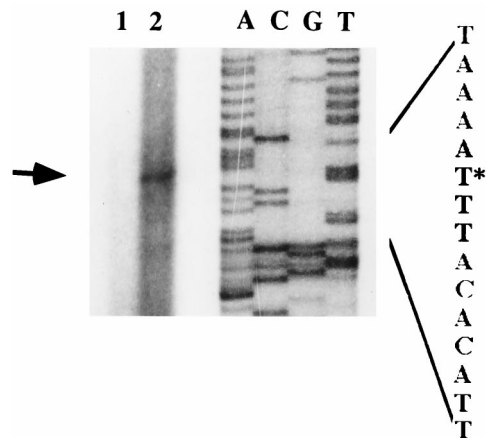


FIG. 3. Primer extension analysis of RNA from strain O395 grown in low-iron medium using a 21-mer oligonucleotide at an annealing temperature of 55°C. Lanes A, C, G, and T are the corresponding lanes of the DNA sequencing ladder. Lane 1 is the control without added primer, and lane 2 is the primer extension reaction. The identified transcriptional start site (arrow) is shown with an asterisk in Fig. 4.

was examined for homology to the Fur binding consensus sequence of *E. coli*. As shown in Fig. 4, the *vibF* promoter region has an area of dyad symmetry that shares 16 of 19 bp with the *E. coli* Fur binding consensus sequence GATAATGATTATT ATTAAC (7, 8, 14, 23).

DNase I footprinting assays. Footprinting studies were performed to document binding of Fur to the predicted *vibF* promoter. When Fur was titrated into the binding reaction mixture, a DNase I footprint was seen overlapping the Fur binding consensus sequence within the *vibF* promoter (Fig. 4 and 5). The footprint was dependent on the presence of metal in the binding mixture (data not shown).

DISCUSSION

The identification of two genes in the vibriobactin uptake apparatus, *viuA*, the ferric vibriobactin outer membrane receptor gene, and *viuB*, a vibriobactin utilization gene, suggested that other genes involved in vibriobactin biosynthesis and uptake might be clustered in the same region of the *V. cholerae* chromosome. A large DNA fragment downstream of *viuB* was

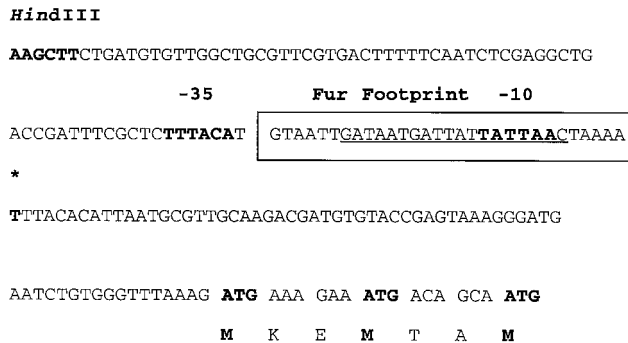


FIG. 4. Nucleotide sequence of the putative promoter region of *vibF*. The deduced amino acid sequence of VibF is shown in single-letter code beneath the DNA sequence. The likely transcriptional start site (*), -10 box (-10), and -35 box (-35) are indicated above the sequence. Three potential starting methionine codons are shown. An inverted repeat homologous to the Fur binding consensus sequence of *E. coli* is underlined. The binding site for Fur deduced from DNase I protection (see Fig. 5) is boxed.

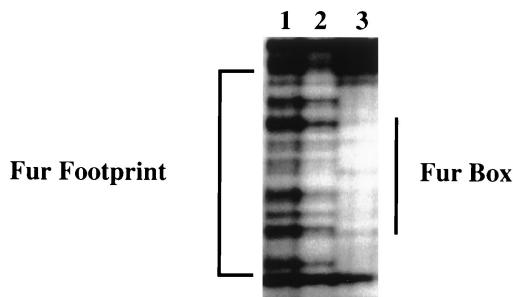


FIG. 5. Various amounts of purified Fur were incubated with a DNA fragment containing the *viuB-vibF* intergenic region (10 nM concentration) and subjected to DNase I digestion. Lanes: 1, DNA fragment alone; 2, DNA fragment incubated with 10 nM Fur; 3, DNA fragment incubated with 50 nM Fur. The DNase I footprint is indicated, as is the location of the Fur binding consensus sequence shown in Fig. 4.

recovered from *V. cholerae* O395. Sequence analysis of the cloned region revealed a single large open reading frame which extended beyond the recovered chromosomal DNA, suggesting that the entire coding region is very large and encodes a protein greater than 2,158 amino acids in length. Complementation studies demonstrated that the cloned fragment, despite encoding a truncated protein, restored the ability of an *E. coli entF* mutant to grow in low-iron medium. Given the complementation of *entF* function, the open reading frame was named *vibF*.

The construction of a stable, in-frame *vibF* mutant by in vivo marker exchange and the effect of introduction of the amino-terminal 2,158 amino acids of VibF in *trans* on the phenotype of the mutant provided evidence that *vibF* encodes a protein involved in ferric vibriobactin biosynthesis. Cross-feeding studies demonstrated that *V. cholerae vibF* mutant strain JRB18 was unable to synthesize vibriobactin but utilized the exogenous siderophore normally; the clone of the 5' region of *vibF* in *trans* restored the ability of the mutant to produce vibriobactin. The mutant phenotype of JRB18 is unlikely to be caused by polar effects on other genes downstream of *vibF* in an operon, since the deletion of *vibF* in JRB18 is in frame and the complementing chromosomal fragment on pJRB20 contains only *viuB* in addition to the cloned fragment of *vibF*.

Many genes encoding proteins involved in siderophore biosynthesis and uptake are themselves regulated by iron, and the suggested role of *vibF* in vibriobactin biosynthesis is supported by the observation that *vibF* is negatively regulated by iron at the transcriptional level. Analysis of *vibF* promoter activity in different mutant strains, using an operon fusion of the *vibF* promoter to *E. coli phoA*, revealed that *vibF* expression is under the control of the *fur* gene but is unaffected by the expression of *irgA* or *viuA*. The biological significance of the twofold reduction of *vibF* expression observed in a strain mutant in the positive transcriptional regulator IrgB is unclear. In prior studies, gel shift assays demonstrated that IrgB does not bind to the *vibF* promoter region (50), suggesting at most an indirect role of IrgB in *vibF* expression. Analysis of the promoter region of *vibF* identified a region homologous to the *E. coli* repressor protein Fur binding consensus sequence. The footprint of purified *V. cholerae* Fur overlapping this consensus sequence in the *vibF* promoter further supported the hypothesis that the transcriptional regulation of *vibF* by iron is under the control of Fur.

The very large size of the deduced VibF protein is unusual, as it is twice the size of the previous largest open reading frame reported for *V. cholerae* (27); such large open reading frames

are not common in bacterial genomes in general. One class of proteins that are very large are the multifunctional nonribosomal peptide synthetases, which are multisubunit enzymes ranging in size from 100 to over 1,600 kDa (46). These proteins, produced mainly by soil bacteria and filamentous fungi, synthesize bioactive peptides "nonribosomally" on a protein template. Evaluation of the deduced amino acid sequence of VibF revealed significant homology with many members of this protein class.

Peptide synthetases are multienzyme complexes comprising one or more modules in a single, large polypeptide chain; each module represents a functional unit and acts as an independent enzyme to catalyze the sequential activation and condensation of one amino or hydroxy acid into the peptide product. The specific linear order of the modules determines the final sequence of the linked amino acids in the product. Each module shares homology with the others in specific functional domains (46). The most common module characterized in bacteria contains about 600 amino acids, with an approximately 500-amino-acid acyladenylation domain, essential for amino acid recognition and activation, and a 100-amino-acid domain involved in thioester formation. Up to nine core sequence motifs have been identified in the acyladenylation domain, with an additional core motif within the thioester domain (29, 38, 46). The serine residue of the thioester motif (LGXSI) is the binding site for the cofactor 4'-phosphopantetheine and is essential for thioester formation. Between modules are elongation domains, which contain spacer motifs (HHXXXDG), involved in acyl transfer. In the multiple-carrier model, each amino acid is activated as an aminoacyl adenylate and linked to the enzyme as a thioester with a phosphopantetheinyl group. Elongation then occurs by transfer of the activated carboxyl to the amino group of the next amino acid, allowing stepwise condensation (13).

Six core sequence motifs, along with a spacer motif, are present in the VibF sequence (Fig. 2), suggesting that the truncated protein encoded on pJRB20 comprises one complete functional module of a peptide synthetase. The enterobactin synthetases EntE (2,3-dihydroxybenzoate AMP ligase) (42) and EntF (a serine-activating enzyme) (39) also belong to the family of adenylate-forming enzymes. We speculate that the identified module in VibF may act to link 2,3-DHBA to threonine in the production of vibriobactin in *V. cholerae*, but this proposed function does not explain the way in which pJRB20 complements the *entF* mutation in AN117, as it seems unlikely that VibF would activate serine to form enterobactin in *E. coli*. Wyckoff et al. were able to complement the *entF* mutation in AN117 with a cosmid clone containing portions of the different vibriobactin gene cluster they identified; however, the siderophore produced was clearly novel, as it did not comigrate with either enterobactin or vibriobactin in chromatographic analysis (52). This gene cluster contains a gene, *vibH*, which encodes a protein similar to the amino-terminal region of EntF (GenBank accession no. U52150). It may be that both VibH and VibF complement an *entF* mutation in *E. coli* by directing the synthesis of variant siderophores.

The linkage of *viuA-viuB-vibF* to *vibB-vibE-vibC-vibA-vibH-viuP-viuD-viuG-viuC-vibD* has been previously investigated (52). A probe from the *viuA* gene did not hybridize with cosmid clones containing the *vibB-vibD* region, and the cosmids did not complement a *viuA* mutant strain. We believed that the *vibB-vibD* region was unlikely to lie upstream of *viuA*, as 3 kbp of chromosomal DNA upstream of *viuA* has been recovered from *V. cholerae* O395 and found to contain the genes *glxX*, *ompA*, and *orfA*, a gene without database homology (GenBank accession no. AF030977) (W. J. Liao, M. H. Choi, and J. R.

Butterton, 33rd Joint Conf. Cholera Relat. Diarrheal Dis., p. 179, 1997). However, since several members of the family of nonribosomal peptide synthetases are extremely large, reaching over 10,000 bp in length, it remained possible that *vibF* was linked downstream to *vibB-vibD*. However, preliminary sequence data from The Institute for Genomic Research suggests that these regions, although both on the *V. cholerae* large chromosome, are widely separated. The observation that a *vibF* mutation eliminates siderophore biosynthesis argues against the possibility that *V. cholerae* possesses two separate but redundant siderophore production and utilization clusters.

The identified extent of VibF represents one of the longest proteins identified in gram-negative enteric pathogens, being similar in size to HMWP2 of *Yersinia* spp. (2,035 amino acids), which also likely functions as a nonribosomal peptide synthetase in siderophore biosynthesis (2, 22). Analysis of preliminary sequence data from The Institute for Genomic Research of a different *V. cholerae* strain, O1 El Tor N16961, suggests that the complete *vibF* open reading frame encodes a 2,413-residue protein, and the complete open reading frame contains only one identified biosynthetic module. There are no additional open reading frames in the region downstream of *vibF* that encode proteins with homology to nonribosomal peptide synthetases or siderophore biosynthetic enzymes. If the biosynthetic module identified in VibF acts to link a single 2,3-DHBA molecule to threonine in the production of vibriobactin, other proteins, perhaps encoded by the *vibB-vibD* region, may complete the synthesis of vibriobactin by linking another molecule of 2,3-DHBA to norspermidine via a threonine residue, along with a third 2,3-DHBA molecule attached directly to the norspermidine backbone. Further characterization of the vibriobactin biosynthetic genes will allow a detailed understanding of the biosynthetic steps that are needed for production of this siderophore.

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