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Cloning, Sequencing, and Transcriptional Regulation of *viuA*, the Gene Encoding the Ferric Vibriobactin Receptor of *Vibrio cholerae*

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A 74-kDa iron-regulated outer membrane protein of *Vibrio cholerae* acts as the receptor for the *V. cholerae* iron-siderophore complex, ferric vibriobactin. MBG14, a mutant of *V. cholerae* 0395 containing a Tn*phoA* insertion in a gene designated *viuA*, lacks this 74-kDa outer membrane protein and is unable to bind or utilize exogenous ferric vibriobactin. Introduction of a plasmid containing the complete *viuA* coding sequence and 513 bp of upstream DNA into MBG14 restored ferric vibriobactin utilization to the mutant. The DNA insert in this plasmid was sequenced, revealing a single open reading frame of 2,061 bp, encoding a deduced protein of 687 amino acids with a predicted molecular mass of 76,417 Da and a predicted initial signal sequence of 37 amino acids. ViuA showed only weak homology to two iron-regulated outer membrane proteins in *Escherichia coli*, IutA and FecA. Construction of *viuA*::Tn*phoA* gene fusions allowed study of the regulation of *viuA* expression by iron. This regulation in *E. coli* was dependent on the *fur* gene. Northern (RNA) blot analysis of RNA from wild-type *V. cholerae* grown in high- and low-iron media revealed a monocistronic *viuA* message that was negatively regulated by iron at the transcriptional level. Primer extension analysis identified a single transcriptional start site, located 243 bp above the translational start site. The promoter region of *viuA* contained two interrupted dyad symmetric nucleotide sequences, overlapping the −10 and −35 boxes, each similar to the *E. coli* Fur binding consensus sequence. Another iron-regulated gene in *V. cholerae* that is negatively regulated by *fur*, *irgA*, requires a positive transcriptional activator (*irgB*) for expression. However, a strain of *V. cholerae* mutant in *irgB* was unaffected in *viuA* expression. These studies suggest that there is conserved, global coordinate iron regulation in *V. cholerae* by *fur*; additional regulatory factors, superimposed upon the *fur* system, may provide more precise control of individual iron-regulated genes.

Vibrio cholerae, like several other gram-negative pathogenic organisms, has developed elegant methods to survive in the inhospitable low-iron environment of the human host. Iron is an essential element for bacterial growth and survival (6, 9, 10) but is not freely available under aerobic conditions. In order to compete with a human host's own high-affinity iron binding proteins, transferrin and lactoferrin, *V. cholerae* has evolved several distinct iron transport systems. Like many other bacteria (10, 29, 34), *V. cholerae* produces a siderophore—a low-molecular-weight iron chelator—which binds iron with high affinity and allows it to be solubilized and transported into the cell (18). The *V. cholerae* phenolate-type siderophore, vibriobactin, is similar in structure to the *Escherichia coli* siderophore, enterobactin, and the *Agrobacterium tumefaciens* siderophore, agrobactin (18). Vibriobactin is not, however, required for bacterial survival; mutants in vibriobactin synthesis or transport can alternatively use ferric citrate (39) or resort to a heme-iron transport system (42). The hemolysin of *V. cholerae* may be used to lyse intestinal epithelial cells during colonization, thereby obtaining iron directly from heme.

Genes involved in iron uptake are themselves regulated by iron. Regulation of gene expression by iron has been most thoroughly studied in *E. coli*. In this organism, low-iron conditions induce production of the siderophores enterobactin and aerobactin (10, 31), as well as the cognate outer membrane receptors for the iron-siderophore complexes, FepA and IutA (30, 33). Expression of these and other proteins that are necessary for iron uptake is regulated by a single locus, *fur*, whose protein product represses gene transcription in the presence of sufficient iron (2, 7, 8, 11, 12, 19, 20).

The regulation of iron uptake in *V. cholerae* is less well defined. The production of both vibriobactin and hemolysin is negatively regulated by iron (42), as is the production of a number of outer membrane proteins (37–39). The 77-kDa major iron-regulated outer membrane protein has been shown to be a virulence factor of *V. cholerae* and is encoded by the gene *irgA* (17). IrgA is homologous to the class of TonB-dependent outer membrane transport proteins in *E. coli* (14), but mutants in *irgA* do not lack any of the characterized mechanisms of iron uptake in *V. cholerae*, including the utilization of ferric vibriobactin (14).

We have previously reported that the 74-kDa iron-regulated outer membrane protein of *V. cholerae*, encoded by the gene *viuA*, functions as the *V. cholerae* ferric vibriobactin receptor (41). MBG14, a mutant of *V. cholerae* 0395 with a Tn*phoA* insertion in *viuA*, lacks the 74-kDa outer membrane protein and is unable to bind or utilize exogenous ferric vibriobactin, while synthesizing increased amounts of vibriobactin and transporting ferrichrome and ferric citrate normally. Introduction of a plasmid containing the complete *viuA* coding sequence and 513 bp of upstream DNA into MBG14 restores the wild-type phenotype to the mutant (41).

Both positive and negative regulatory elements play roles in the expression of *V. cholerae* iron-regulated proteins. A mutant of *V. cholerae*, which constitutively synthesizes vibriobactin and hemolysin in the presence of iron, is complemented to normal iron regulation by a plasmid containing

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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
Strains		
V. cholerae		
0395	Sm^r	25
MBG14	*viuA*::Tn*phoA* Sm^r Km^r	17
MBG259	*irgB*::pMBG111 Sm^r Ap^r	16
JRB1	*viuA*::Tn*phoA irgB*::pMBG111 Sm^r Km^r Ap^r	This study
E. coli		
CC118	Δ(*ara-leu*) *7697* Δ(*lac*)*X74 araD139 phoA*Δ20 *galE galK thi rpsE rpoB argE*(Am) *recA1*	24
SM10 λ *pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ *pirR6K* Km^r	28
DHB24	Δ(*ara-leu*)*7697 araD139* Δ(*lac*)*X74 galE galK rpsL thi malF*Δ*3 phoA*Δ*Pvu*II *phoR zad*::Tn*10 pcnB*/F′ *lac pro lacI*^{q} Sm^r Tc^r	7
SBC24	*fur*::Tn*5* derivative of DHB24, Sm^r Tc^r Km^r	7
Plasmids		
pMBG111	Suicide plasmid with 676-bp internal *Hin*cII-*Bgl*II fragment of *irgB*, Ap^r	16
pJRB7	pUC18 with 1.8-kbp *Hin*dIII insert of *V. cholerae* 0395 chromosomal DNA, containing 1.3 kbp of 5′ end of *viuA* and 513 bp of upstream DNA; Ap^r	This study
pJRB8	pBR322 with 1.8-kbp *Hin*dIII insert from pJRB7, Ap^r	This study
pJRB9,13	pJRB8, *viuA*::Tn*phoA* Ap^r Km^r	This study
pJRB15	pUC18 with 2.7-kbp *Hin*dIII-to-*Sac*I fragment of *V. cholerae* 0395 chromosomal DNA, containing entire coding sequence of *viuA*; Ap^r	This study

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance, Tc^r, tetracycline resistance.

the *E. coli fur* gene (42). Litwin et al. have recently cloned and sequenced the *fur* gene from *V. cholerae* and shown that it is functionally and structurally homologous to the *fur* gene of *E. coli* (23). The transcription of *irgA* in *V. cholerae* is negatively regulated by iron, and the promoter of this gene contains a 19-bp dyad symmetric nucleotide sequence that is homologous to Fur binding sites in *E. coli* (15), suggesting that this gene may be negatively regulated by the *fur* gene of *V. cholerae*. Unlike iron-regulated genes in *E. coli*, however, transcription of *irgA* also requires a positive transcriptional activator protein, IrgB. *irgB* is divergently transcribed from *irgA*, and the promoters of *irgA* and *irgB* overlap the same Fur box; the transcription of *irgB* is itself negatively regulated by iron (16).

In this report, we describe the cloning and sequencing of *viuA*, the gene encoding the ferric vibriobactin receptor of *V. cholerae*. We present evidence that *viuA* is negatively regulated by iron at the transcriptional level. Primer extension localized the promoter of *viuA*, revealing two interrupted dyad symmetrical sequences overlapping the promoter, each similar to the *E. coli* Fur binding consensus sequence. Using a *viuA*::Tn*phoA* gene fusion, we determined the roles of Fur and IrgB in the regulation of *viuA* by iron.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1, with the exception of plasmids pJRB1 through pJRB6, which are described in detail below and are depicted in Fig. 1. *E. coli* DH5α was obtained from Bethesda Research Laboratories Life Technologies, Inc. (Gaithersburg, Md.). Standard plasmid cloning vectors pBR322, pUC18, and pUC19 were from laboratory stocks.

Media. All strains were maintained at −70°C in Luria broth (LB) media (36) containing 15% glycerol. LB solidified with agar was used for high-iron solid media. Low-iron solid media were prepared by treating LB with Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.) as described previously (17). For *V. cholerae* strains, Chelex-LB agar was supplemented with 0.3% glucose. Ampicillin (100 μg/ml), kanamycin (45 μg/ml), streptomycin (100 μg/ml), 5-bromo-4-chloro-3-indolyl phosphate (XP) (Amresco, Solon, Ohio; 40 μg/ml), or X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; International Biotechnologies, Inc., New Haven, Conn.; 40 μg/ml) was added as appropriate.

Tris-buffered medium (T medium) (40), with and without the addition of 36 μM $FeSO_4$, was used for growth in high- and low-iron conditions, respectively. For *V. cholerae* strains, T medium was supplemented with 0.4% sucrose; for *E. coli* strains, the medium contained thiamine (10 μg/ml) and the L amino acids arginine and leucine (40 μg/ml).

Assays. Utilization of vibriobactin was determined by bioassay (41). Organisms (10^4/ml) of indicator bacterial strains were solidified in iron-depleted media (LB agar with 75 μg of ethylenediamine di[*o*-hydroxyphenylacetic acid] per ml). The ability of these strains to use vibriobactin was determined by measuring the growth of the indicator strains around 10-μl spots of stationary-phase bacterial cultures (producer strains) after incubation at 37°C for 18 h. The indicator strains would not grow in the absence of usable exogenous siderophore or iron. Solutions of ferrous sulfate (1 mM) and vibriobactin (2 mM) were used as positive controls.

Regulation of Tn*phoA* gene fusions by iron was assessed by measuring alkaline phosphatase activities of strains grown in low- versus high-iron media. Strains were grown overnight in LB media, diluted 1:100 into T media with or without added $FeSO_4$, and again grown overnight. Measuring the amount of hydrolysis of *p*-nitrophenyl phosphate (Amresco) by permeabilized cells allowed calculation of alkaline phosphatase activity, as previously described (26). Screening for colonies with a $PhoA^+$ phenotype under low-iron conditions was performed by streaking strains onto Chelex-LB agar containing the chromogenic substrate XP. $PhoA^+$ colonies are blue on XP, while $PhoA^-$ colonies are white.

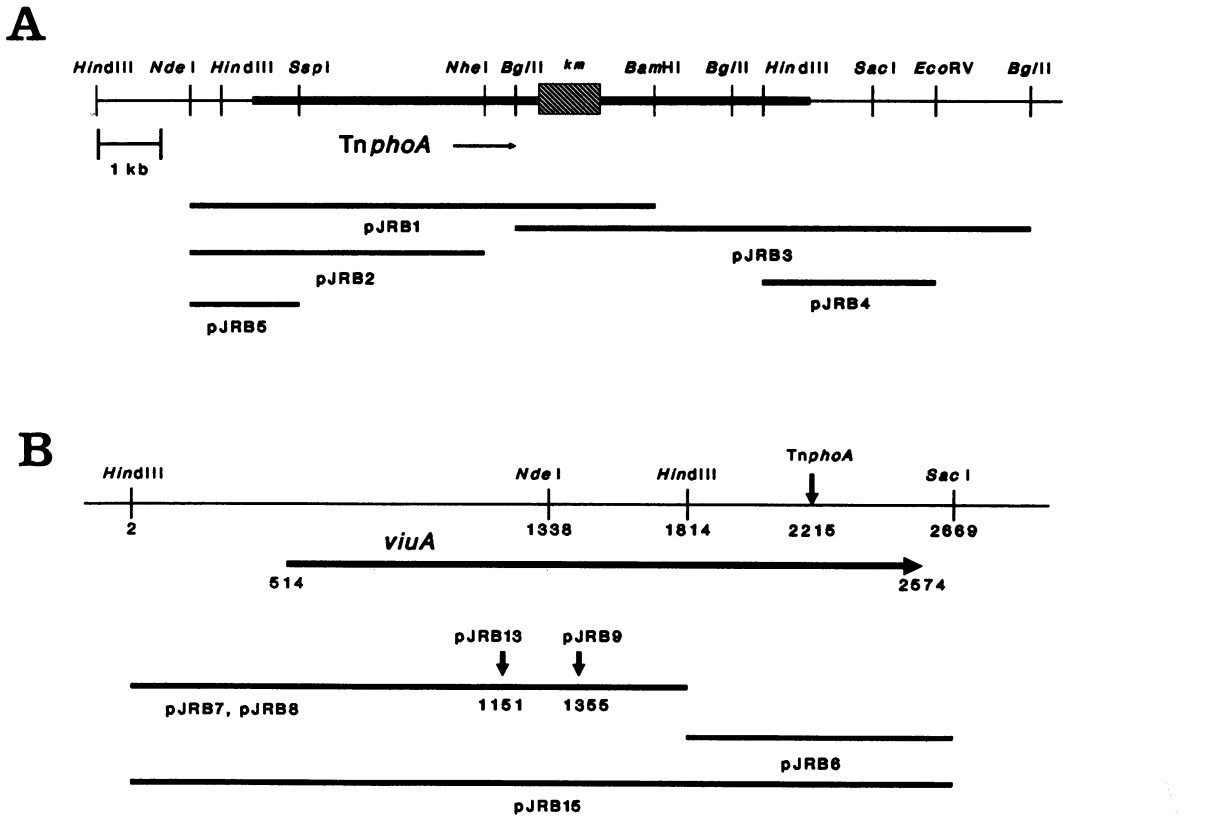

FIG. 1. (A) Partial restriction map of MBG14 chromosomal DNA, with relevant restriction enzyme sites and location of cloned fragments. The thick solid bar represents Tn*phoA* DNA. The arrow indicates the direction of transcription of *phoA*. The hatched box indicates the location of the kanamycin resistance marker in Tn*phoA*. The construction of plasmid clones is described in the text. (B) Partial restriction map of 0395 chromosomal DNA. The location of the Tn*phoA* insert in MBG14 is indicated with a vertical arrow. The locations of relevant restriction enzyme sites are numbered to correspond to the complete nucleotide sequence (Fig. 2). The extent of the coding region of *viuA* is indicated by the solid arrow. The construction of plasmid clones is described in the text. The locations of the Tn*phoA* insertions in pJRB9 and pJRB13 are indicated by the vertical arrows above the pJRB8 chromosomal fragment.

Genetic methods. Isolation of plasmid and bacterial chromosomal DNA, preparation of RNA, restriction enzyme digests, agarose gel electrophoresis, colony blot hybridization, Southern hybridization of DNA separated by electrophoresis, and Northern (RNA) blot analysis were performed according to standard molecular biologic techniques (36). GeneScreen Plus and Colony/PlaqueScreen hybridization transfer membranes (DuPont Biotechnology Systems, NEN Research Products, Boston, Mass.) were used according to the manufacturer's protocols for Southern, Northern, and colony blot hybridizations. DNA sequencing was performed with the Sequenase DNA Sequencing Kit (United States Biochemical Corporation, Cleveland, Ohio). For Northern blot analysis, an equal amount of RNA, calculated from the optical density at 260 nm, was loaded into each lane. Primer extension analysis was performed as previously described (27); oligonucleotide primers were hybridized to RNA in 0.4 M NaCl and 40 mM PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)], pH 6.4, without formamide, at 60°C for 2 h. RNasin and avian myeloblastosis virus reverse transcriptase were obtained from Bethesda Research Laboratories Life Technologies, Inc.

Plasmids were transformed into *E. coli* strains by standard techniques or were electroporated into *V. cholerae* or *E. coli* strains by using a Gene Pulser (Bio-Rad Laboratories) according to the manufacturer's protocol and modified for electroporation into *V. cholerae* as previously described (16). Electroporation conditions were 2,500 V at 25-μF capacitance, producing time constants of 4.7 to 4.9 ms.

DNA restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, and calf intestinal alkaline phosphatase were used according to the manufacturers' specifications. Restriction enzyme-digested chromosomal DNA fragments were separated on 1% agarose gels; appropriate size fragments were cut from the gel under UV illumination and purified by electroelution (36) or the freeze-squeeze technique (44). DNA fragments used as probes were radiolabelled with [α-^{32}P]dCTP by using a random priming labelling kit (Prime Time C Oligonucleotide Labelling Biosystem; International Biotechnologies, Inc.), or by oligonucleotide [γ-^{32}P]ATP end labelling, using standard techniques (36).

Construction of plasmids. Mapping of the chromosomal Tn*phoA* insertion in MBG14 was performed by Southern hybridization of restriction enzyme-digested chromosomal DNA, using as a probe an internal fragment of Tn*phoA* (1.8 kbp in length, from the *Hin*dIII site at bp 3,111 to the *Bam*HI site at bp 4,973) which had been labelled by random priming. The resulting restriction map (data not shown) revealed a unique *Nde*I site approximately 900 bp upstream of the fusion joint. MBG14 chromosomal DNA was digested to completion with *Nde*I, which does not cut within Tn*phoA*, and *Bam*HI; the single *Bam*HI site in Tn*phoA* is located

downstream of the kanamycin resistance marker and the end of *phoA* (Fig. 1A). Fragments (5 to 7 kbp) were size fractionated by gel electroelution and ligated into the unique *Nde*I and *Bam*HI sites of pBR322. The ligation mix was electroporated into CC118, and colonies containing the desired clone were distinguished by selection for ampicillin resistance (carried on pBR322) and kanamycin resistance (carried on Tn*phoA*). Recovered plasmids contained identical 6-kbp chromosomal inserts; the construct was confirmed by restriction mapping and was named pJRB1 (Fig. 1A). For ease of subcloning, pJRB1 was reduced in size by deletion of the 2.3-kbp fragment from *Nhe*I in Tn*phoA* (at bp 2,931) to the unique *Nhe*I site in pBR322 to create pJRB2. pJRB5 was created from pJRB2 by deletion of the 3.2-kbp *Ssp*I fragment (from bp 171 in Tn*phoA* to bp 4,170 in pBR322).

Recovery of chromosomal DNA downstream of the fusion joint with Tn*phoA* proceeded in a similar manner. MBG14 chromosomal DNA was partially digested with *Bgl*II (for 15 min at 37°C) to allow recovery of the 8-kbp fragment from the upstream *Bgl*II site in Tn*phoA* to the downstream chromosomal *Bgl*II site that would contain the kanamycin resistance marker of Tn*phoA* (Fig. 1A). Following digestion, 6.0- to 8.5-kbp fragments were size fractionated by gel electroelution and ligated into the unique *Bam*HI site of pBR322. The ligation mix was electroporated into CC118, and colonies were again selected for ampicillin and kanamycin resistance. The recovered plasmid, pJRB3, contained a 6.6-kbp chromosomal insert, with 2.3 kbp of downstream chromosomal DNA fused to the terminal 4.3 kbp of Tn*phoA* (Fig. 1A). pJRB4 was constructed by ligating the 2.1-kbp *Hin*dIII-to-*Eco*RV chromosomal fragment of pJRB3 into the unique *Hin*dIII and *Sma*I sites of pUC19.

The chromosomal DNA flanking the Tn*phoA* insertion was sequenced from the above constructs and their derivatives. This sequence revealed an open reading frame extending from upstream of the *Nde*I site (877 bp above the fusion joint) to a termination codon 359 bp downstream of the fusion with Tn*phoA*. Oligonucleotide probes, internal to this open reading frame, were synthesized in order to recover this region from wild-type 0395 chromosomal DNA, to ensure that no sequence alterations had occurred because of the insertion of the transposon. Recovery of additional upstream chromosomal DNA was performed as follows. Southern hybridization analysis of *Hin*dIII-digested 0395 chromosomal DNA, probed with an end-labelled synthetic oligonucleotide probe internal to the upstream open reading frame, revealed a *Hin*dIII site 1.3 kbp upstream of the *Nde*I site (Fig. 1B). Wild-type *V. cholerae* 0395 chromosomal DNA was digested with *Hin*dIII; DNA fragments from 1.6 to 2.0 kbp were size selected and ligated into the *Hin*dIII site of pUC18. The ligation mixture was electroporated into DH5α and plated onto LB agar containing ampicillin and X-Gal. Colonies containing inserts lack β-galactosidase activity and are white on these indicator plates. White colonies were transferred to nitrocellulose discs by using the colony blot technique and probed with the end-labelled oligonucleotide. Colonies identified in this manner contained identical 1.8-kbp inserts; the construct, named pJRB7, was confirmed by restriction mapping and Southern hybridizations (data not shown).

Recovery of chromosomal DNA from wild-type *V. cholerae* 0395 that encompassed the region of the Tn*phoA* fusion in MBG14 was performed in a similar manner. To recover the expected 850-bp fragment from *Hin*dIII (401 bp above the fusion joint) to *Sac*I (454 bp below the fusion), 0395 chromosomal DNA was digested with *Sac*I and *Hin*dIII. Fragments from 650 bp to 1.2 kbp in size were selected and ligated into the unique *Sac*I and *Hin*dIII sites of pUC18. The ligation mixture was electroporated into DH5α and plated onto LB containing ampicillin and X-Gal; white colonies were transferred to nitrocellulose and probed by using a labelled oligonucleotide internal to the open reading frame downstream of the *Hin*dIII site. The identified colonies contained a plasmid with the expected 850-bp fragment, which was confirmed by restriction mapping and Southern hybridizations and named pJRB6.

Construction of a single plasmid containing the entire coding sequence of *viuA* in wild-type *V. cholerae*, from the upstream *Hin*dIII site to the *Sac*I site downstream of the termination codon, was performed as follows. pJRB7 was digested with *Hin*dIII, and the 1.8-kbp chromosomal fragment was separated from the parent plasmid by gel electrophoresis. This fragment was purified and then ligated into the unique *Hin*dIII site of pJRB6, and the ligation mixture was transformed into DH5α. Resulting colonies containing plasmids with the 1.8-kbp *Hin*dIII fragment inserted in the correct orientation were identified by restriction mapping; the desired plasmid was named pJRB15. The complete sequence of the chromosomal insert in pJRB15 was established either by double-stranded sequencing of the recovered plasmids or by single-stranded sequencing of fragments subcloned into appropriate M13 vectors and by using either universal primers or synthetic oligonucleotides to allow complete sequencing of both DNA strands.

Construction of plasmid-borne *viuA*::Tn*phoA* gene fusions. pJRB7 was digested with *Hin*dIII, and the 1.8-kbp chromosomal fragment was separated from the parent plasmid by gel electrophoresis. This fragment was purified and ligated into the unique *Hin*dIII site of pBR322, and the ligation mixture was transformed into CC118. Colonies containing plasmids with the 1.8-kbp *Hin*dIII fragment inserted in the inverse orientation to the tetracycline promoter were identified by restriction mapping; the resulting plasmid was named pJRB8.

The transposon Tn*phoA* was introduced into pJRB8 as previously described (24). pJRB8 was transformed into competent CC118(F'42 *lacI3 zzf-2*::Tn*phoA*); transformants were plated onto LB with ampicillin and kanamycin (30 μg/ml). A suspension of overnight growth from this plate was made in LB broth and plated onto LB containing ampicillin and kanamycin (300 μg/ml). Transposition of Tn*phoA*, which encodes kanamycin resistance, from the low-copy-number F' plasmid onto the high-copy-number pJRB8 is selected by the step up in concentration of kanamycin. Overnight growth from this plate was suspended in LB broth, and plasmid DNA was prepared. The plasmid mixture was transformed into a fresh CC118 background, and transformants were selected on LB containing ampicillin, kanamycin (30 μg/ml), and XP. Doubly resistant, blue colonies contain in-frame fusions of Tn*phoA* to secreted gene products encoded on pJRB8. Several Tn*phoA* insertions within *viuA* on pJRB8 were localized by restriction mapping. Two plasmids, pJRB9 and pJRB13, containing the two Tn*phoA* insertions most proximal in *viuA*, were selected for further study. The fusion joints between *viuA* and Tn*phoA* in pJRB9 and pJRB13 were subcloned into M13mp18, and the nucleotide sequences across the fusions were determined.

Construction of JRB1. *V. cholerae* JRB1, which contains an insertion mutation in *irgB*, was constructed from strain MBG14 in the following manner. SM10 λ *pir* containing pMBG111, a suicide plasmid which contains an internal

TABLE 2. Utilization of various iron sources by wild-type and mutant *V. cholerae*[a]

Indicator strain	Zone of growth (mm) around producer strains or iron-containing compounds				
	0395	MBG14	MBG14(pJRB15)	Fe	Vibriobactin
0395	26	20	24	16	20
MBG14	0	0	0	15	0
MBG14(pJRB15)	25	19	25	16	21

[a] The indicator strains were seeded into low-iron agar under conditions such that no growth occurs in the absence of usable exogenous siderophore or iron. The numbers represent sizes of zones of growth of the indicator organisms in the presence of different producer strains, iron, or vibriobactin.

fragment of *irgB* (16), was conjugated with MBG14, and colonies that were doubly resistant to streptomycin (encoded by MBG14) and ampicillin (encoded by pMBG111) were selected. pMBG111 is unable to replicate in MBG14, because this strain does not contain the *pir* gene; doubly resistant colonies occur, therefore, by homologous recombination between the internal fragment of *irgB* on pMBG111 and the corresponding chromosomal gene in MBG14, causing insertional inactivation of *irgB*. To confirm the construction in JRB1, we purified chromosomal DNA from strains 0395, MBG259 (a previously constructed insertion mutation of pMBG111 into the *irgB* gene of 0395), MBG14, and JRB1. The chromosomal DNAs were cut with *Eco*RV, an enzyme that does not cut within either *irgB* or pMBG111. Southern hybridization of these digests, probed with an internal fragment of *irgB*, demonstrated the increase in size of the recognized fragment expected for integration of pMBG111 into *irgB* in both MBG259 and JRB1 (data not shown).

DNA and protein data base searches. Nucleotide and derived amino acid sequences were analyzed with IntelliGenetics Suite Software (IntelliGenetics, Inc., Mountain View, Calif.) by using the data bases of the GenBank On-Line Service. The FASTA algorithm for protein homology (32) was used to compare the deduced protein product to sequences in the Swiss-Protein data bases (version 18; University of Geneva). The hydropathicity index profile was calculated by using the Kyte-Doolittle formula (22).

Nucleotide sequence accession number. The GenBank accession number for the complete *viuA* sequence presented here is M90461.

RESULTS

Ferric vibriobactin utilization bioassay. Analysis of the ability of MBG14(pJRB15) to utilize exogenous ferric vibriobactin was evaluated in a bioassay. As shown in Table 2, the mutant MBG14 was unable to utilize exogenous ferric vibriobactin supplied by strains producing this siderophore. In contrast, strain MBG14(pJRB15) demonstrated growth comparable to that of the wild-type parent with ferric vibriobactin as the sole iron source. These results confirm that the *viuA* clone in pJRB15 can complement the mutation in MBG14 in *trans*.

Nucleotide sequence of *viuA*. The 2.7-kbp chromosomal region in pJRB15, from the upstream *Hin*dIII site to the downstream *Sac*I site, was cloned and sequenced as described above. The complete nucleotide sequence is presented in Fig. 2. A single open reading frame of 2,061 bp is present, beginning at bp 514 and ending at bp 2,574. A Shine-Dalgarno sequence is present upstream of the starting methionine. An inverted repeat, suggestive of a transcription terminator, begins 25 bp beyond the termination codon of the coding sequence.

Protein analysis of ViuA. The deduced amino acid sequence of ViuA is shown below the nucleotide sequence in Fig. 2. The gene encodes a protein of 687 amino acids and has a predicted molecular mass of 76,417 Da. The predicted pI is 4.59.

(i) Hydropathicity index profile. The hydropathicity plot of ViuA suggests an initial 37-amino-acid signal sequence, with a 17-amino-acid hydrophobic domain spanning residues 20 to 36. There are no further areas of localized hydrophobicity in the remainder of ViuA to suggest transmembrane regions (data not shown). The molecular mass of the mature protein, following cleavage of the predicted 37-amino-acid signal sequence, would be 72,412 Da. This correlates closely with the 74-kDa iron-regulated outer membrane protein lost in MBG14, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (41).

(ii) Homology of ViuA to *E. coli* iron-regulated outer membrane proteins. The FASTA algorithm for protein homology was used to compare ViuA with other proteins in the Swiss-Protein data base. Weak homologies were noted only with IutA, the *E. coli* aerobactin outer membrane protein receptor (16.7% identity in a 407-amino-acid overlap), and FecA, the *E. coli* dicitrate outer membrane receptor (14.9% identity in a 616-amino-acid overlap). No significant homologies were found to FepA, IrgA, or OM2, the outer membrane receptor for ferric anguibactin in *Vibrio anguillarum* (1).

Iron regulation of *viuA*::Tn*phoA* on the chromosome of MBG14. Regulation by iron of the *viuA*::Tn*phoA* fusion in MBG14 was assessed by measuring alkaline phosphatase activity after growth in iron-limited and iron-replete media (Table 3). There was a significant increase in alkaline phosphatase activity in low-iron conditions compared with that in high-iron conditions, with an induction ratio of 184 in T medium with and without added $FeSO_4$.

Iron regulation of *viuA*::Tn*phoA* cloned on a plasmid. We were unable to recover the *viuA*::Tn*phoA* fusion directly from the chromosome of MBG14. Therefore, we introduced Tn*phoA* into *viuA* on a plasmid clone by transposition. Creating gene fusions between the promoter and proximal portions of *viuA*, carried on the plasmid pJRB8, and the gene for bacterial alkaline phosphatase, on Tn*phoA*, allowed a quantitative assessment of *viuA* expression. Four distinct in-frame fusions between *viuA* and Tn*phoA* were identified; the location of Tn*phoA* within each was determined by restriction mapping. The two most proximal insertions, within pJRB9 and pJRB13, were confirmed by sequence analysis of the fusion joints, and their locations in the *viuA* sequence are indicated in Fig. 1B.

Plasmids pJRB9 and pJRB13 were transformed into *E. coli* CC118, which carries a deletion of its own chromosomal *phoA* gene. Alkaline phosphatase activity of the *viuA*::Tn*phoA* fusions was measured after growth in T medium with or without added iron. As shown in Table 3, both fusions demonstrated highly expressed and iron-regulated alkaline phosphatase activity, suggesting that the *cis*-acting regions necessary for the expression and iron regulation of *viuA* are localized to the region of cloned chromosomal DNA in pJRB8. The lower induction ratios in low- and high-iron media between CC118(pJRB9) or CC118 (pJRB13) and MBG14 may be the result of the high copy number of *viuA* carried on the plasmids or less efficient regulation of a *V. cholerae* gene in an *E. coli* background. The fact that DHB24(pJRB13), a strain with a *pcnB* mutation

FIG. 2. Nucleotide sequence of *viuA*, from the upstream *Hin*dIII site to the *Sac*I site downstream of the termination codon. The locations of relevant restriction enzyme sites are indicated, as is the location of the Tn*phoA* insertion in MBG14 (▼). The deduced amino acid sequence of ViuA is shown in three-letter code below the DNA sequence. The transcriptional start site (*), −10 box (**−10**), and −35 box (**−35**) are indicated below the line, and the Shine-Dalgarno sequence (**SD**) is indicated above the line. A 27-bp interrupted dyad symmetric sequence overlapping the −35 box and a 23-bp interrupted dyad symmetric sequence overlapping the −10 box are indicated by inverted horizontal arrows above the sequence. The termination codon of ViuA (. . .) is shown; after it is an inverted repeat suggestive of a transcription terminator (horizontal arrows below the sequence).

that lowers plasmid copy number, had an induction ratio similar to that of CC118(pJRB13) (Table 3) suggests that plasmid copy number may be less important than lower affinity of the heterologous Fur protein in explaining the different induction ratios for *viuA*::Tn*phoA* seen between *V. cholerae* and *E. coli*.

Regulation of *viuA* expression by iron in *E. coli* Fur$^+$ and Fur$^-$ backgrounds. To evaluate the role of the Fur protein in the iron regulation of *viuA* expression, pJRB13 was transformed into *E. coli* DHB24 and SBC24, which are isogenic except for a *fur*::Tn*5* mutation in SBC24. Alkaline phosphatase activities of DHB24(pJRB13) and SBC24(pJRB13) when grown in low- and high-iron media are shown in Table 3. Expression of *viuA* is normally regulated by iron in the Fur$^+$ background but is constitutively expressed in the Fur$^-$

TABLE 3. Alkaline phosphatase activities in T media with and without added iron

Strain	Alkaline phosphatase activity (U/A_{600} of cells) in T medium	
	Without added iron	With 36 μM iron added
MBG14	184	1
CC118(pJRB8)	1	1
CC118(pJRB9)	687	30
CC118(pJRB13)	514	53
DHB24(pJRB13)	52	4
SBC24(pJRB13)	125	118
JRB1	159	2

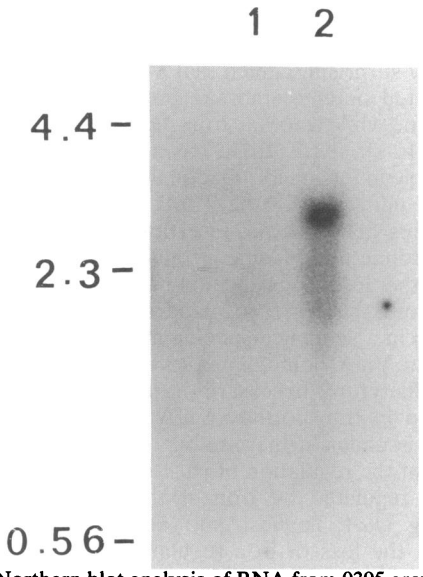

FIG. 3. Northern blot analysis of RNA from 0395 grown in high- and low-iron media, probed with a ^{32}P-labelled fragment of *viuA*. Lane 1, 0395 grown in high iron; lane 2, 0395 grown in low iron. The positions of double-stranded DNA molecular mass markers (in kilobase pairs) are indicated on the left. Additional Northern blots, run against single-stranded RNA molecular mass markers, were used to derive the estimated 2.5-kb size of the RNA transcript.

strain, demonstrating that, in an *E. coli* background, regulation of *viuA* expression by iron is dependent on the *fur* gene.

Regulation of *viuA* expression in *V. cholerae* IrgB$^+$ and IrgB$^-$ backgrounds. Iron regulation of the cloned *viuA* gene in an *E. coli* background suggests either that no additional *V. cholerae* genes are needed for *viuA* regulation or that such additional genes are also present in *E. coli* and can complement their absence in the *viuA* clone. One gene that is present in *V. cholerae* and important in iron regulation is *irgB*, the positive transcriptional activator of the *V. cholerae* virulence gene *irgA*. To investigate the role of *irgB* in *viuA* expression in *V. cholerae*, we constructed strain JRB1 by integration of the suicide plasmid pMBG111 into the *irgB* gene of MBG14; this technique has been previously used to construct *irgB* mutations of *V. cholerae* (16). The construction of JRB1 was confirmed by Southern hybridization of digested chromosomal DNA, as described in Materials and Methods.

Alkaline phosphatase activities of the *viuA*::Tn*phoA* fusions in MBG14 and JRB1 were compared under low- and high-iron conditions, as shown in Table 3. Expression and iron regulation of alkaline phosphatase activity in JRB1 is similar to that in MBG14, indicating that *irgB* is not involved in the regulation of *viuA* expression.

Northern blot analysis of transcriptional regulation of *viuA* by iron. To determine the size of the RNA transcript encoding *viuA* in *V. cholerae* and to investigate if regulation of *viuA* expression by iron occurs at the transcriptional level, we performed Northern blot analysis of RNA prepared from *V. cholerae* 0395 following growth in low- and high-iron media. The blot was probed with a radioactively labeled fragment internal to the open reading frame of *viuA*. A single band of approximately 2.5 kb in size was seen in RNA prepared from 0395 grown in low-iron conditions; no bands were seen in RNA following growth in high-iron conditions (Fig. 3), indicating that transcription of *viuA* is negatively

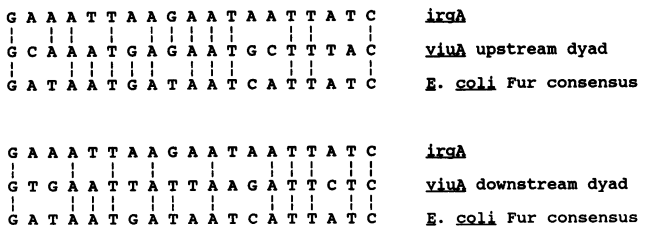

FIG. 4. Homology between the proposed iron regulatory regions of *viuA*, *irgA*, and the Fur consensus sequence of *E. coli* (7, 8, 11, 12). The upstream dyad overlaps the proposed −35 box of the *viuA* promoter; the downstream dyad overlaps the proposed −10 box. The *viuA* dyads shown are the central parts of the larger interrupted dyad symmetric sequences shown in Fig. 2.

regulated by iron. The presence of approximately equal amounts of RNA in the high- and low-iron lanes was confirmed by probing identical parallel lanes with a radioactively labeled synthetic oligonucleotide complementary to a constitutively expressed metabolic gene of *V. cholerae*, glyceraldehyde 3-phosphate dehydrogenase (4) (data not shown). The size of the transcript, compared with the size of the *viuA* open reading frame and transcriptional stop site, suggests that the *viuA* transcript is monocistronic and that the transcriptional start site is located several hundred base pairs upstream of the translational start site.

Localization of the transcriptional start site and promoter of *viuA* by primer extension. Primer extension analysis with RNA from strain 0395 grown in low-iron medium was performed with two synthetic oligonucleotides complementary to DNA sequences located 112 bp upstream and 13 bp downstream of the methionine start codon of *viuA* (data not shown). Primer extensions with each oligonucleotide identified the same likely transcriptional start site, located 243 bp above the translational start site, as indicated in Fig. 2. The −10 box of the *viuA* promoter, located 7 bp upstream of the likely transcriptional start site, and the −35 box, both homologous to the *E. coli* consensus sequences (21), are indicated in Fig. 2. A 23-bp interrupted dyad symmetric sequence overlapping the −10 box was identified, and a 27-bp interrupted dyad symmetric sequence overlapping the −35 box was located, as indicated by horizontal arrows in Fig. 2.

Homology of the *viuA* promoter region to Fur binding consensus sequences. The two interrupted dyad symmetric sequences in the *viuA* promoter were examined for homology to the Fur consensus sequence of *E. coli* (7, 8, 11, 12) and to the dyad symmetric sequence located in the promoter region of *irgA* (15). As shown in Fig. 4, the central parts of both dyad symmetric sequences in the *viuA* promoter are similar both to the *E. coli* Fur consensus and to the proposed iron regulatory region of *irgA*. Of 19 bp in the central part of each dyad in the *viuA* promoter, 12 are identical to the 19-bp *E. coli* Fur consensus.

DISCUSSION

Sequence analysis of *viuA* and the effect of introduction of *viuA* in *trans* on the phenotype of the *V. cholerae* mutant MBG14 provide evidence suggesting that *viuA* is the structural gene for the 74-kDa iron-regulated outer membrane protein missing in this mutant. Complementation studies described previously (41) demonstrate that *viuA* in *trans* restores the 74-kDa iron-regulated outer membrane protein lost in the mutant. Since the introduced chromosomal frag-

ment on pJRB15 contains only a single open reading frame, it is unlikely that the mutation in MBG14 produced by Tn*phoA* insertion is due to a polar effect of the transposon on a nearby gene rather than by insertion into the structural gene for the 74-kDa outer membrane protein itself. In addition, the molecular mass of the mature protein predicted from the ViuA open reading frame is 72,417 Da, corresponding in size to the 74-kDa iron-regulated outer membrane protein lost in MBG14 and restored by the introduction of *viuA* in *trans*. Finally, ViuA has an initial signal sequence consistent with an exported protein, as would be expected for an outer membrane protein; hydropathicity analysis demonstrates that the signal sequence of ViuA follows the model of procaryotic exported protein signals, with a positively charged amino terminal region, a 17-amino-acid hydrophobic core, and a typically conserved signal peptidase cleavage site (Ala-Tyr-Ala, residues 35 to 37) (45, 46). The signal sequence of ViuA is unusual in its overall length of 37 amino acids and in the 11-residue hydrophobic region that precedes the N-terminal positively charged residues. However, analysis of procaryotic signal sequences has demonstrated that the N-terminal region is the most variable of the signal sequence domains, both in terms of length and amino acid composition (46).

This report also presents evidence supporting the hypothesis that the 74-kDa outer membrane protein, ViuA, acts as the *V. cholerae* ferric vibriobactin receptor. The insertion mutation by Tn*phoA* in *viuA* prevents utilization of exogenous ferric vibriobactin, which is restored by *viuA* in *trans*. Our previous studies demonstrated that ferric vibriobactin binding to purified outer membrane proteins was also decreased in the mutant MBG14 and was likewise restored by *viuA* in *trans*. A *viuA*::Tn*phoA* fusion, as seen in strain MBG14, produces a PhoA$^+$ phenotype (17). Hybrid proteins encoded by in-frame fusions between a target gene and *phoA* have alkaline phosphatase activity if the PhoA portion of the fusion is transported into the periplasmic space, identifying proteins that are expressed at the cell surface (24). Hydropathicity analysis of ViuA reveals an initial signal sequence but no other localized hydrophobic regions consistent with transmembrane domains. This structure is not suggestive of a cytoplasmic membrane protein but would be consistent with a periplasmic or outer membrane protein. Examination of the carboxy terminus of ViuA reveals a potential amphipathic β-sheet with a carboxy-terminal phenylalanine and hydrophobic residues at positions 3, 5, 7, and 9 from the carboxy terminus. This sequence motif is highly conserved among outer membrane proteins of gram-negative bacteria but has not been found in periplasmic proteins (43). The specific loss of an iron-regulated outer membrane protein in MBG14 and the restoration of this protein with *viuA* in *trans* are most consistent with the hypothesis that ViuA is an outer membrane protein and functions as the ferric vibriobactin outer membrane receptor.

Comparison of the amino acid sequence of ViuA with those of other sequenced iron-regulated outer membrane proteins revealed only weak homologies to IutA, the *E. coli* aerobactin outer membrane receptor, and to FecA, the *E. coli* dicitrate outer membrane receptor; no significant homology was found to FepA, the *E. coli* ferric enterobactin receptor. The absence of homology between ViuA and FepA is perhaps not unexpected, given the differences in structure of their respective siderophores (18). However, what does seem unusual is the apparent absence in ViuA of a TonB box, the conserved region in the amino-terminal segment of TonB-dependent outer membrane transport proteins that is the putative binding site of TonB (3). Most of these proteins are also iron regulated. The lack of a TonB box in ViuA was particularly surprising, given that ViuA appears to act as an iron-regulated outer membrane protein receptor for ferric vibriobactin, while another iron-regulated outer membrane protein of *V. cholerae*, IrgA, has a TonB box but plays no apparent role in ferric vibriobactin utilization (14). However, a study of mutations in the TonB boxes of BtuB and Cir demonstrates that only the insertion of the turn-promoting residues proline and glycine strongly affects transport ability; most other insertions had no functional effect, suggesting that the TonB box is recognized as a local conformation rather than in a sequence-specific manner (3). The lack of a sequence in ViuA homologous to the TonB box of *E. coli* does not, therefore, necessarily rule out a role for a TonB-like protein in transport by ViuA in *V. cholerae*; such a possibility is under further study.

Studies of the regulation of *viuA* demonstrate that *viuA* is negatively regulated by iron at the transcriptional level. Introducing *viuA*::Tn*phoA* into an *E. coli* Fur$^-$ mutant resulted in the loss of iron regulation of *viuA* expression, providing evidence that *viuA* is negatively regulated by Fur. The testing of *viuA* expression in a *V. cholerae* Fur$^-$ background awaits the construction of an appropriate mutant in this organism. The recent cloning of the *V. cholerae fur* gene by Litwin et al. (23) should help direct this construction.

Analysis of the promoter region of *viuA* revealed two interrupted dyad symmetric nucleotide sequences that were similar to the *E. coli* Fur binding consensus sequence. Although the homology between the dyads in the *viuA* promoter region and the *E. coli* Fur consensus is not perfect—12 of 19 bp in the central part of each dyad are identical to the *E. coli* Fur consensus—this consensus has been formed from the analysis of only a small number of promoter regions (7, 8, 11, 12). Additional sequence information may identify which sites are of critical importance in Fur binding and may reveal differences between the *E. coli* Fur box and *V. cholerae* Fur binding regions. Two contiguous Fur binding sites, one overlapping the −35 region and one overlapping the −10 region, have been reported previously for the aerobactin operator of *E. coli* (12), and a pair of contiguous, sequentially occupied Fur binding sites have been identified in the 5′ untranslated region of the *E. coli* enterobactin biosynthetic gene *entC* (5). Perhaps the presence of two potential Fur binding sites in the *viuA* promoter, although each less homologous to the consensus than previously identified genes, may contribute to the tight regulation by iron of *viuA* expression seen in *V. cholerae*. Further experiments to directly test this hypothesis are under way.

The long, 243-bp untranslated leader of *viuA* is similar to long, untranslated leader regions of other iron-regulated genes. The *E. coli* enterobactin system includes two bidirectional iron-regulated control regions, located within the *fepA-fes* and *fepB-entC* intercistronic regions (5, 33). *fepB* mRNA contains a 214-nucleotide leader which contains multiple inverted repeat sequences (5). Similarly, long leader transcripts have been described for both *fes* and *fepA* mRNA; several potential stem-loop secondary structures are also observed in these leaders (33). It has been proposed that the secondary structures of these leaders could be involved in stability of the mRNA or translational efficiency (5). Experiments are in progress to evaluate similar roles for the long *viuA* leader in regulating *viuA* expression.

That additional genes could play a role in the expression of *viuA* is suggested by the regulatory systems present for

several other iron-regulated genes. Expression of the *V. cholerae* virulence gene *irgA* involves not only negative regulation of transcription but also a requirement for a positive transcriptional activator gene, *irgB* (16). The *V. anguillarum* iron-regulated anguibactin iron-uptake system requires *angR*, which is a positive regulator of the uptake system that is itself negatively regulated by iron at the transcriptional level (35), but this gene has not been found to be divergently transcribed from any genes which it is known to regulate (13). We wished to determine if regulation of expression of *viuA* by iron similarly requires any additional factors.

The normal regulation of *viuA* expression by iron in an *E. coli* background suggested either that no additional *V. cholerae* genes were needed for *viuA* regulation or that such genes, if needed, were supplied in *trans* by *E. coli*. Alkaline phosphatase assays of JRB1, an *irgB* mutant of MBG14, suggest that *irgB* does not play a role in the regulation of *viuA* expression by iron. Analysis of the predicted amino acid sequence encoded by the cloned 270-bp fragment located upstream of the transcriptional start site of *viuA* did not reveal another divergently transcribed open reading frame. The possibility that other *trans*-acting elements, shared with *E. coli*, are involved in the regulation of *viuA* expression is under further study.

The observation that *viuA* is regulated by *fur* supports the hypothesis that there is conserved, global coordinate iron regulation by *fur* in pathogenic microorganisms. Additional regulation, superimposed upon the *fur* system, may be provided by accessory factors, such as *irgB* and *angR*; such additional factors may provide the fine-tuning needed for precise control of individual iron-regulated virulence genes.

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