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Identification of the *Vibrio cholerae* Enterobactin Receptors VctA and IrgA: IrgA Is Not Required for Virulence

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The gram-negative enteric pathogen *Vibrio cholerae* requires iron for growth. *V. cholerae* has multiple iron acquisition systems, including utilization of heme and hemoglobin, synthesis and transport of the catechol siderophore vibriobactin, and transport of several siderophores that it does not itself make. One siderophore that *V. cholerae* transports, but does not make, is enterobactin. Enterobactin transport requires TonB and is independent of the vibriobactin receptor ViuA. In this study, two candidate enterobactin receptor genes, *irgA* (VC0475) and *vctA* (VCA0232), were identified by analysis of the *V. cholerae* genomic sequence. A single mutation in either of these genes did not significantly impair enterobactin utilization, but a strain defective in both genes did not use enterobactin. When either *irgA* or *vctA* was supplied on a plasmid, the ability of the *irgA vctA* double mutant to use enterobactin was restored. This indicates that both VctA and IrgA transport enterobactin. We also identify the genes *vctPDGC*, which are linked to *vctA* and encode a periplasmic binding protein-dependent ABC transport system that functions in the utilization of both enterobactin and vibriobactin (VCA0227-0230). An *irgA*::Tn*phoA* mutant strain, MBG40, was shown in a previous study to be highly attenuated and to have a strong colonization defect in an infant mouse model of *V. cholerae* infection (M. B. Goldberg, V. J. DiRita, and S. B. Calderwood, Infect. Immun. 58:55-60, 1990). In this work, a new *irgA* mutation was constructed, and this mutant strain was not significantly impaired in its ability to compete with the parental strain in infant mice and was not attenuated for virulence in an assay of 50% lethal dose. These data indicate that the virulence defect in MBG40 is not due to the loss of *irgA* function and that *irgA* is unlikely to be an important virulence factor.

Vibrio cholerae causes the severe diarrheal disease cholera (18, 32). Many of the genes required for this gram-negative pathogen to cause disease in humans or in animal models have been described. A key virulence factor is the cholera toxin, which is responsible for the severe voluminous diarrhea characteristic of cholera. The toxin-coregulated pilus is essential for colonization of the intestinal epithelium (65). The synthesis of this bundle-forming, type IV pilus is coordinately regulated with the synthesis of cholera toxin, and proper transcriptional control of this regulon is required for virulence. Another *V. cholerae* gene that is reported to be required for virulence is *irgA*. The *irgA*::Tn*phoA* mutant strain MBG40 has a decreased competitive index and nearly a 100-fold-increased 50% lethal dose (LD_{50}) in an infant mouse model relative to its parental strain O395 (24). Other genes required for full virulence have been identified more recently, and these include genes for nutrient acquisition, stress response, and proper colonization of the lower small intestine (see references 18 and 32 for reviews).

Genes for the acquisition of the nutrient iron play a critical role in the ability of a pathogen to establish and maintain an infection in its host (48). A variety of strategies for iron acquisition have evolved in pathogenic bacteria. These include the synthesis and secretion of small iron-chelating molecules termed siderophores (8, 12, 17). After binding iron in the extracellular environment, the iron-siderophore complex is transported back into the cell, where the iron is removed and used for various cellular functions. Another strategy for iron acquisition is the direct use of host iron compounds, including heme, hemoglobin, transferrin, and lactoferrin. Uptake of siderophores and iron from host compounds involves specific, high-affinity outer membrane receptors. The energy for transport of these ligands across the outer membrane is provided by the TonB-ExbBD complex, which transduces energy from the inner membrane (7). Transport through the periplasm and across the inner membrane is facilitated by a periplasmic binding protein-dependent ABC transport system. In these systems the periplasmic binding protein binds the ligand and delivers it to the inner membrane permease. This permease usually consists of two integral inner membrane proteins, each of which is bound to an ATPase subunit. The hydrolysis of ATP by the ATPase subunit provides the energy for transport across the inner membrane (8, 17).

Multiple iron acquisition systems have been identified in *V. cholerae*. Heme and hemoglobin are efficiently used as iron sources (28, 29, 40, 44, 61). *V. cholerae* makes and transports the catechol siderophore vibriobactin (10, 25, 33, 34, 38, 68, 69) and can use several siderophores that it does not make, including ferrichrome (25), enterobactin (70), and schizokinen (57).

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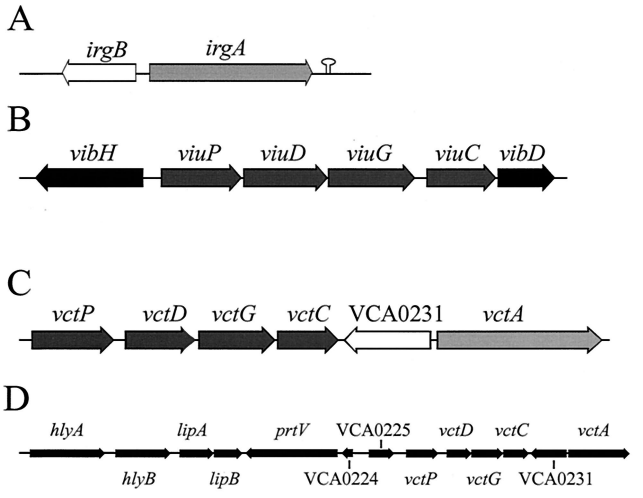

FIG. 1. Genetic maps of siderophore transport genes in *V. cholerae*. The arrows indicate the direction of transcription for each of the ORFs. For unnamed reading frames, the locus designation from the TIGR database is used (27). The scale is the same for parts A to C, while part D is drawn on a smaller scale. (A) The *irgA irgB* region, showing a potential rho-independent terminator downstream of *irgA* (21, 22); (B) the *viuPDGC* region (70); (C) the *vct* region (27); (D) the *vct* region, showing linkage of these siderophore transport genes with an iron-regulated hemolysin gene and other genes possibly involved in tissue damage (27, 45).

The vibriobactin biosynthesis genes are located in two separate gene clusters, one of which also contains the vibriobactin outer membrane receptor gene, *viuA* (10, 11, 60). The other vibriobactin biosynthesis gene cluster contains the genes for a periplasmic binding protein-dependent ABC transport system. These proteins, ViuPDGC, function in the utilization of both vibriobactin and enterobactin. Although *V. cholerae* strains carrying mutations in either *viuP* or *viuG* had a reduced ability to use vibriobactin and enterobactin, the utilization of these siderophores could still be detected. This suggested that an additional system for the transport of catechol siderophores across the inner membrane is present in *V. cholerae* (70).

The outer membrane receptor has been identified for ferrichrome (52) but not for schizokinen or enterobactin. A candidate outer membrane receptor for transport of one of these iron complexes is IrgA. The predicted amino acid sequence of IrgA has homology to those of TonB-dependent outer membrane receptors, and it is an abundant iron-regulated protein in the outer membrane (21). Its expression is negatively regulated by the general iron regulatory protein Fur and is positively regulated by the divergently transcribed upstream gene *irgB* (22, 23) (Fig. 1A). These data suggested that IrgA may function in the transport of iron into the cell. However, no defect in the utilization of various iron compounds was detected in the *irgA* mutant, and thus no ligand for this potential receptor was identified (21).

In this work we identified *irgA* and *vctA* as the two enterobactin receptor genes present in *V. cholerae*. We also determined that genes linked to *vctA* constitute the second periplasmic binding protein-dependent ABC transport system for vibriobactin and enterobactin. Contrary to previous reports, we found that a newly constructed *irgA* mutant competed efficiently with its wild-type parent for colonization in an infant mouse model of *V. cholerae* virulence. In infant mice, the LD_{50} of this *irgA* mutant was the same as the LD_{50} of its wild-type parent.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
V. cholerae		
CA401	Classical biotype	20
CA40130	Vibriobactin synthesis mutant of CA401	25
CA40130N	Nal^r derivative of CA40130	40
ARM316	CA40130N *irgA*::*cam*	This study
AGO310	CA40130N *vctA*::*kan*	This study
ARM616	CA40130N *irgA*::*cam, vctA*::*kan*	This study
O395	Classical biotype	39
ARM516	O395 *irgA*::*cam*	This study
MBG40	O395 *irgA*::Tn*phoA*	24
Lou15	El Tor biotype	58
EWV103	Lou15 *viuP*::*cam*	70
EWV108	Lou15 *vctPD*::*kan*	This study
EWV109	Lou15 *viuP*::*cam, vctPD*::*kan*	This study
N16961	El Tor biotype	J. Kaper
E. coli		
DH5α	Cloning strain, Ent^+	26
SY327(λpir)	Host for pGP704 derivatives	41
SM10(λpir)	Host for pGP704 derivatives	41
Plasmids		
pBluescript SK−	Cloning vector	Stratagene
pWKS30	Cloning vector	66
pACYC184	Cloning vector	15
pMTLcam	*cam* resistance gene	67
pUC4K	*kan* resistance gene	Pharmacia
pACYCsac	pACYC184 containing *sacB*	44
pHM5	pGP704 carrying *sacB*	53
pVIB147	*viuPDGC* from Lou15 in pWKS30	70
pCAT119	*vctA* from CA401 in pWKS30	This study
pCAT120	*vctPDGC* from Lou15 in pWKS30	This study
pCAT121	*irgBA* from CA401 in pWKS30	This study

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and siderophore utilization assays. The strains and plasmids used in this study are listed in Table 1. The iron chelator ethylenediamine di(*ortho*-hydroxyphenylacetic acid) (EDDA) was deferrated by the method of Rogers (51). The antibiotic concentrations used were 250 μg of carbenicillin per ml, 50 μg of kanamycin per ml, and 50 (for *E. coli*) or 5 (for *V. cholerae*) μg of chloramphenicol per ml. The bioassay for siderophore utilization was performed as previously described (69).

DNA sequencing. DNA was sequenced using an Applied Biosystems Prism 377 DNA sequencer (Perkin-Elmer Corp.). Amino acid sequence alignments were performed using the ClustalW feature of the MacVector package (46). The BLAST program (2) was used to search the National Center for Biotechnology Information protein database.

Plasmid construction. To construct pCAT119, the *vctA* gene of CA401 was amplified with *Pfu* DNA polymerase by using primers CatAfor2 (5′-TGGTGG TCATCACATCGCAATC) and CatArev (5′-TGTTTCATCCCAAGTCGCAG G). The resulting PCR product was cloned into the *Eco*RV site of pWKS30. pCAT120 was constructed by PCR amplification of the *vctPDGC* genes from strain Lou15 with *Pfu* DNA polymerase by using primers Liz180 (5′-GCCAAA CCATTGCGGAAATAGAAG) and Liz181 (5′-CGTTATCTCAGCACCAAG AGGGAC). The product was cloned into the *Sma*I site of pWKS30. To construct pAMR18, a fragment containing *irgA* and *irgB* was PCR amplified from the strain CA401 by using *Pfu* DNA polymerase and primers irgBA1 (5′-AGTGAATTC

AGCTAAAGAACTGGTGG) and irgBA2 (5′-GGGAATTCTAACCGATACT CTAGGC). The PCR product was digested with *Eco*RI and cloned into the *Eco*RI site of pACYC184. The *irgBA* insert was moved as an *Eco*RI fragment to pWKS30 to yield pCAT121.

Mutant strain construction. To construct EWV108 and EWV109, the region carrying *vctPDGC* was amplified with *Taq* polymerase by using the primers Liz 174 (5′-TCGGTCACAAAGAGGGGATAGG) and Liz 175 (5′-ATTGCGAAG TAACAGCGAGAGG) with Lou15 DNA as a template. The product was cloned into pGEM-Teasy (Promega) to yield pCAT114. The *kan* cassette from pUC4K was inserted to replace the internal *Eco*RV-*Msc*I fragment to yield the plasmid pCAT116. The *Not*I fragment containing the *vct* genes was subcloned into pACYCsac to give pCAT117. Allelic exchange mutations were obtained as previously described (69) in Lou15 to give EWV108 or in EWV103 to give EWV109. For the construction of AGO310, the *vctA* region was amplified using primers CatAfor (5′-TCCATTGCTCAGATTGTCCCTC) and CatArev (listed above) with *Taq* polymerase. The product was cloned into pGEM-Teasy to give pCAT103. The *kan* cassette from pUC4K was inserted into the *Sma*I site to give pAGO-cat1. The *Sal*I-*Sph*I fragment was then cloned into *Sal*I-*Sph*I-digested pHM5, and allelic exchange was performed as previously described (40). To make ARM316, ARM516, and ARM616, the *cam* cassette from pMTLcam was inserted as a *Sma*I fragment into the *Sma*I site of the *irgA* gene carried on pAMR18. The *irgA*::*cam* insert was moved as a Klenow-blunted *Cla*I fragment into pHM5 digested with *Eco*RV to yield pAMS5. Allelic exchange was performed as previously described (40) in CA40130N to yield ARM316, in O395 to yield ARM516, and in AGO310 to yield ARM616.

Virulence assays. In vivo competition assays were performed by a protocol modified from that of Taylor et al. (65). Five-day-old prestarved BALB/c mice were inoculated intragastrically with 50 μl of saline containing 0.02% (wt/vol) Evan's blue dye and 10^5 CFU of each strain grown to mid-log phase at 37°C. After 24 h, the mice were sacrificed, and the intestines were isolated and homogenized in sterile phosphate-buffered saline. Serial dilutions were plated on selective or differential media to determine the viable counts for each strain. To calculate the competitive index, the ratio of mutant to wild-type bacteria recovered from the intestine was determined and then normalized by dividing by the ratio of mutant to wild-type bacteria in the initial inoculum. The LD_{50} of each strain was assessed by orally inoculating groups of four 5- to 7-day-old CD-1 mice (Charles River) with a series of 10-fold dilutions of bacteria that had been grown overnight at 30°C in Luria-Bertani broth with a starting pH of 6.5. The inoculum ranged from approximately 10×10^4 through 10×10^8 bacteria. The LD_{50} is based on the extrapolated dose that would have resulted in a mean survival rate of 50% of the mice after 48 h (49).

Nucleotide sequence accession number. The GenBank accession number for the sequence of the *V. cholerae* strain CA401 *vctA* gene is AY061945.

RESULTS

Identification of enterobactin receptors in *V. cholerae*. We previously showed that *V. cholerae* uses the catechol siderophore enterobactin and that enterobactin transport is independent of the vibriobactin receptor ViuA (70). The recent sequencing of the *V. cholerae* genome (27) allowed us to identify candidate enterobactin receptors. The genome contains several open reading frames (ORFs) that potentially encode TonB-dependent outer membrane receptors, and the predicted amino acid sequence of each of these ORFs was used to perform a BLAST search (2) of the National Center for Biotechnology Information nonredundant database. Enterobactin receptors were among the highest-scoring proteins in the BLAST search for two of the predicted *V. cholerae* TonB-dependent receptors, IrgA and a previously uncharacterized protein which we have designated VctA (for *Vibrio* catechol transport) (see below).

irgA was originally identified as the site of the Tn*phoA* insertion in a strain with reduced virulence in an infant mouse model (24). This putative virulence factor has sequence homology with TonB-dependent outer membrane receptors but no identified ligand. In a BLAST search using IrgA, the top-scoring protein with an identified ligand is the *Escherichia coli* enterobactin receptor FepA (21) (Table 2). This suggested that enterobactin might be the ligand for IrgA. To test this, an *irgA* insertion mutation was constructed in CA40130N, a vibriobactin synthesis mutant derived from the classical strain CA401 (40). The vibriobactin mutation was included in the genetic background to reduce the background growth in enterobactin utilization assays. The *irgA* mutant, ARM316, had no significant defect in enterobactin utilization (Table 3). In addition, ARM316 used vibriobactin, ferrichrome, and heme as efficiently as the parental strain CA40130N (Table 3). This result is in agreement with data for the previously characterized *irgA* mutant, MBG40, which also used enterobactin, vibriobactin, ferrichrome, and heme as efficiently as its parent (reference 21 and unpublished data).

The second enterobactin receptor candidate gene, *vctA*, is located in a region containing other potential iron transport genes (Fig. 1C). In a BLAST search, VctA showed the highest homology scores with the *Neisseria gonorrhoeae* enterobactin receptor FetA and also with heme receptors from a variety of organisms (Table 2). A mutation in *vctA* was constructed by allelic exchange, and the mutant was tested for the ability to use enterobactin. As shown in Table 3, the *vctA* mutant strain, AGO310, used enterobactin as well as the parent strain did. Although VctA also has homology with heme receptors, AGO310 used heme efficiently (Table 3), and our studies of heme utilization in *V. cholerae* indicated that VctA is not one of the heme receptors (40).

The failure of both the *irgA* and *vctA* single mutants to show a defect in enterobactin utilization could be due to functional redundancy of these transport systems. To test this, we constructed an *irgA vctA* double mutant. Unlike either of the single mutants, the double mutant strain, ARM616, was completely defective in enterobactin utilization (Table 3). In complementation studies, a plasmid encoding either *irgA* (pCAT121) or *vctA* (pCAT119) was introduced into the double mutant strain. The ability to use enterobactin was restored when either the *vctA* or the *irgA* gene was supplied on a plasmid (Table 3). This is strong evidence that both IrgA and VctA transport enterobactin in *V. cholerae*.

In The Institute for Genomic Research (TIGR) genomic database for *V. cholerae* El Tor strain N16961, the *vctA* ORF (VCA0232) contains a frameshift mutation and should not encode an active protein (27). Our genetic data, however, suggested that *vctA* encodes a functional protein in CA40130N, a derivative of classical strain CA401. To resolve this apparent discrepancy, the sequence of the entire *vctA* gene from CA401 was determined. The CA401 *vctA* sequence contained an additional G residue not present in the published sequence. The *vctA* gene containing this additional residue is predicted to encode a full-length receptor protein. To determine whether a functional *vctA* gene is present in other *V. cholerae* strains, the region of *vctA* containing the additional G was sequenced from the classical strain O395 and from the El Tor strains Lou15 and N16961. In each of these strains, the G which restored the ORF was present, suggesting that a functional *vctA* gene is widely distributed among *V. cholerae* strains (data not shown). It is unclear why our sequence for N16961 *vctA* did not match the sequence in the database.

Characterization of a periplasmic binding protein-depen-

TABLE 2. Homologies of *V. cholerae* enterobactin transport proteins to selected proteins

Protein (ORF)[a] and homolog	Ligand	Identity (%)	Similarity (%)	Reference(s)
IrgA (VC0475)				
E. coli IreA	Unknown	36	56	54
E. coli CirA	Unknown	36	54	43
E. coli O157:H7 Iha	Unknown	33	48	63
E. coli FepA	Enterobactin	26	43	37
VctA (VCA0232)				
N. gonorrhoeae FetA	Enterobactin	22	39	6, 14
P. shigelloides HugA	Heme	22	41	31
S. dysenteriae ShuA	Heme	22	38	42
Y. enterolitica HemR	Heme	21	38	62
VctP (VCA0227)				
B. subtilis YclQ	Unknown	30	50	36
N. gonorrhoeae FetB	Enterobactin	28	48	14
V. anguillarum FatB	Anguibactin	26	48	1
C. coli CeuE	Enterobactin	25	47	50
V. cholerae ViuP	Vibriobactin	19	35	70
VctD (VCA0228)				
B. subtilis Yc1N	Unknown	43	72	36
C. coli CeuB	Enterobactin	42	69	50
V. anguillarum FatD	Anguibactin	35	66	35
V. cholerae ViuD	Vibriobactin	24	43	70
VctG (VCA0229)				
B. subtilis Yc1O	Unknown	48	69	36
C. coli CeuC	Enterobactin	40	65	50
V. anguillarum FatC	Anguibactin	26	57	35
V. cholerae ViuG	Vibriobactin	22	44	70
VctC (VCA0230)				
B. subtilis Yc1P	Unknown	51	75	36
C. coli CeuD	Enterobactin	43	67	50
V. cholerae ViuC	Vibriobactin	27	51	70

[a] ORF numbering is according to the TIGR database (27).

dent ABC transport system linked to *vctA*. *vctA* is linked to several genes that could also function in iron transport (Fig. 1C). Divergently transcribed from *vctA* is the ORF VCA0231, which has homology to the AraC/XylS family of transcriptional regulators. The function of this gene is not known. Upstream of VCA0231 are four genes with sequences that suggest that they encode a periplasmic binding protein-dependent ABC transport system. The *vctPDGC* genes have the highest homology to the enterobactin transport systems of *N. gonorrhoeae* (14) and *Campylobacter coli* (50) and to the anguibactin transport system of *Vibrio anguillarum* (1, 35) (Table 2). These homologies suggested that VctPDGC might function in the transport of enterobactin and possibly other catechol siderophores across the inner membrane. From these homologies it can be deduced that the *vctP* product is the periplasmic binding protein, the *vctD* and *vctG* products function as the inner membrane permease, and the *vctC* product is the ATPase for the system. A second set of genes, *viuPDGC*, had previously been identified as an ABC transport system for vibriobactin and enterobactin in *V. cholerae* (Fig. 1B) (70).

TABLE 3. IrgA and VctA promote utilization of enterobactin

Indicator strain	Relevant phenotype	Zone of stimulation (mm) with[a]:			
		DH5α (enterobactin)	Lou15 (vibriobactin)	Heme	Ferrichrome
CA40130N	Parent	17	26	18	24
ARM316	$IrgA^-$	14	24	17	22
AGO310	$VctA^-$	16	26	18	23
ARM616/pWKS30	$VctA^-$ $IrgA^-$	0	25	17	28
ARM616/pCAT119	$VctA^+$ $IrgA^-$	16	28	16	26
ARM616/pCAT121	$VctA^-$ $IrgA^+$	16	19	16	25

[a] Cultures of the indicator strains were seeded into L agar containing 100 μg of EDDA per ml. *E. coli* strain DH5α (as a source of enterobactin), *V. cholerae* strain Lou15 (as a source of vibriobactin), heme, or ferrichrome was spotted onto the medium. The zone of growth was measured at 18 h after inoculation.

TABLE 4. ViuPDGC and VctPDGC promote utilization of enterobactin and vibriobactin

Indicator strain	Relevant phenotype	Zone of stimulation (mm) with[a]:		
		DH5α (enterobactin)	Lou15 (vibriobactin)	Ferrichrome
Lou15	Wild type	18	24	28
EWV103/pWKS30	ViuP$^-$	16	12	18
EWV108/pWKS30	VctPD$^-$	17	21	28
EWV109/pWKS30	ViuP$^-$ VctPD$^-$	0	0	17
EWV109/pCAT120	ViuP$^-$ VctPD$^+$	13	20	22
EWV109/pVIB147	ViuP$^+$ VctPD$^-$	12	22	22

[a] Cultures of the indicator strains were seeded into L agar containing 250 μg of EDDA per ml. *E. coli* strain DH5α (as a source of enterobactin), *V. cholerae* strain Lou15 (as a source of vibriobactin), or ferrichrome was spotted onto the medium. The zone of growth was measured at 18 h after inoculation.

Mutations in *viuP* or *viuG* reduced, but did not eliminate, the transport of vibriobactin and enterobactin, indicating the presence of an additional system for the transport of catechol siderophores across the inner membrane.

To determine whether VctPDGC constitute this second catechol siderophore transport system, a chromosomal mutation was created by allelic exchange, in which the 3′ region of *vctP* through the 5′ region of *vctD* was replaced by a kanamycin cassette. This mutation was created both in a wild-type Lou15 background to make the single *vctPD* mutant strain EWV108 and in the *viuP* mutant strain EWV103 to make a *vctPD viuP* double mutant, strain EWV109. As shown in Table 4, the *vctPD* mutant had no significant defect in either enterobactin or vibriobactin utilization, whereas the utilization of vibriobactin was reduced, but not abolished, in the *viuP* mutant. However, the *vctPD viuP* double mutant was completely defective in the utilization of both siderophores. When a plasmid clone containing either the *vctPDGC* transport genes (pCAT120) or the *viuPDGC* genes (pVIB147) was introduced into the double mutant strain, the utilization of both siderophores was restored. This indicates that both systems can function in the transport of both vibriobactin and enterobactin.

As previously observed, the *viuP* mutant strain had a smaller zone of growth around both vibriobactin and ferrichrome than either the wild type or the *vctPD* mutant (Table 4). Since all of the Lou15-derived strains in this experiment produce vibriobactin, a low level of vibriobactin is present throughout the plate. This vibriobactin may withhold iron from the *viuP* mutant, which uses vibriobactin with reduced efficiency. Thus, the *viuP* mutant is likely to be somewhat more iron starved than the wild-type parent, resulting in the smaller zones of growth stimulation around vibriobactin and ferrichrome observed with this mutant.

Virulence assays of *irgA* mutants. It has been reported that the *irgA* mutant MBG40 has reduced virulence in animal models. However, it is unclear why loss of one of two enterobactin receptors would attenuate virulence. To address this question, we used allelic exchange to construct a new, defined *irgA* mutation in the classical *V. cholerae* strain O395, the parent strain of MBG40. The presence of the *cam* cassette within *irgA* in mutant strain ARM516 was confirmed by PCR and by Southern blotting (data not shown). To further characterize the defect in this strain, outer membrane fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A protein band with a molecular weight expected for mature IrgA was observed in Coomassie blue-stained fractions from the wild-type parent O395 but not in those from the *irgA* mutant strain ARM516. The presence of this protein band was restored when *irgA* was supplied to ARM516 on the plasmid pCAT121 (data not shown). As expected, ARM516 used enterobactin in a bioassay (data not shown), and this is likely due to the presence of a functional *vctA* gene in this strain.

The ability of ARM516 to compete with its wild-type parental strain O395 in an infant mouse assay was determined (Table 5). In these experiments, infant mice were given an intragastric inoculation of an equal number of wild-type and mutant bacteria, and the ratio of mutant to wild-type bacteria recovered 24 h later from the intestine was determined by plating on selective media. The competitive index of ARM516 was less than 1, but this reduction in competitive index was not statistically significant. In the same experiment, the previously characterized *irgA* mutant MBG40 had the very low competitive index of 0.12 (Table 5). This is in agreement with the competitive index of 0.11 previously reported for this strain (24). Interestingly, when the *irgA* gene was supplied on a plasmid, the ability of MBG40 to compete with the wild-type parent was not restored. This suggests that the defect in MBG40 is not simply the lack of a functional *irgA* gene.

MBG40 has also been reported to have reduced lethality in a mouse model (24). To test whether the newly constructed *irgA* mutant, ARM516, has a significant reduction in virulence, its LD_{50} in infant mice was determined as described in Materials and Methods. The LD_{50} of ARM516 (8.4×10^5) was nearly identical to that of its wild-type parent O395 (1.0×10^6), indicating that ARM516 is not attenuated for virulence. Thus, it appears that the virulence defect observed for MBG40 is specific to that strain and not a general property of *irgA* mutants.

TABLE 5. In vivo competition assays of wild-type and *irgA* mutants of *V. cholerae*

Strain	Competitive index[a]		No. of mice
	Mean (SD)	Range	
ARM516	0.71 (0.46)	0.51–1.21	8
MBG40	0.12 (0.11)	<0.01–0.38	10
MBG40/pCAT121	0.07 (0.02)	0.04–0.09	4

[a] The competitive index of the indicated strain was determined relative to the wild-type parent O395 in an infant mouse model. The competitive index is the output ratio adjusted for the input ratio of the two competing strains, as described in Materials and Methods.

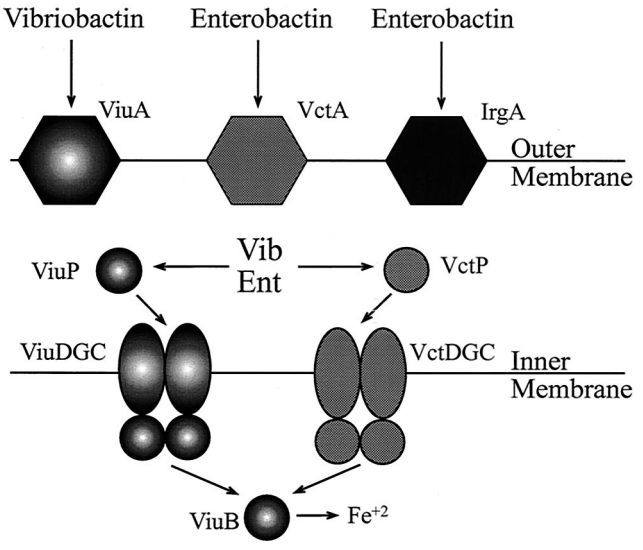

FIG. 2. A model for catechol siderophore transport in *V. cholerae*. Enterobactin can be transported across the outer membrane by either of the two outer membrane receptors, IrgA (VC0475) or VctA (VCA0232), while the endogenous siderophore vibriobactin is transported by ViuA (VC2211). Following entry into the periplasm, both siderophores can be transported across the inner membrane by either the ViuPDGC (VC0776 to VC0779) or the VctPDGC (VCA0227 to VCA0230) system. The iron is then removed from the siderophore by ViuB (VC2210), which has been shown to remove the iron from both enterobactin and vibriobactin (9).

DISCUSSION

We had previously proposed a model for catechol siderophore transport in *V. cholerae* in which ferri-vibriobactin and ferri-enterobactin are each transported by a specific outer membrane receptor (70). Both of these siderophores could then be transported across the inner membrane by either the ViuPDGC or VctPDGC system. Based on the data presented in this work, we have modified our model to show that there are two enterobactin receptor genes, and these are identified as *irgA* (VC0475) and *vctA* (VCA0232) (Fig. 2). An additional system for the transport of catechol siderophores was also identified. These genes, *vctPDGC*, are closely linked to the *vctA* gene (VCA0227-230) (Fig. 1C). The observation that the outer membrane receptors are specific for their ligands while the inner membrane permeases transport a variety of structurally related ligands is reminiscent of the transport of hydroxamate siderophores. In *E. coli*, each hydroxamate siderophore is transported by a specific outer membrane receptor, whereas all are transported across the inner membrane by a single system, FhuBCD (8).

Most of the essential *V. cholerae* genes are encoded on the large chromosome, while the function of many of the genes encoded on the smaller chromosome is unknown. In addition, significant portions of the small chromosome appear to have been acquired by horizontal gene transfer (27). IrgA and ViuPDGC are encoded on the large chromosome, and both of these systems have high homology with the *E. coli* Fep system for enterobactin transport. In contrast, the VctA and VctPDGC genes are encoded on the small chromosome and have homology with enterobactin transport proteins from a diverse group of organisms. These data suggest that *irgA* and *viuPDGC* may have evolved from ancestral *Vibrio fep*-like genes, while the *vctA* and *vctPDGC* genes may have been acquired more recently. The *vct* genes are closely linked to a region encoding several secreted proteins, including an iron-regulated hemolysin, a lipase, and an extracellular protease (45) (Fig. 1D). It was previously proposed that this region could be part of a pathogenicity island promoting host tissue damage and thus aiding in the acquisition of iron and other nutrients by *V. cholerae* (45). The observation that this region is adjacent to siderophore transport genes supports this model.

Enterobactin is the prototype catechol siderophore produced by a group of closely related members of the *Enterobacteriaceae*, including *E. coli*, *Salmonella*, *Klebsiella*, and some strains of *Shigella*. It was recently shown that enterobactin is also produced by the gram-positive bacteria *Streptomyces* spp., suggesting that enterobactin synthesis is more widely distributed phylogenetically than has been previously recognized (19). Many organisms transport siderophores that they do not make, and this may reflect the relative energetic expense of making a siderophore. Enterobactin transport genes have been identified in a number of gram-negative bacteria that do not make this siderophore, including *N. gonorrhoeae* (14), *Bordetella* spp. (5), *Campylobacter* spp. (47, 50), *Pseudomonas aeruginosa* (16), and *Yersinia enterocolitica* (56). In addition, enterobactin transport was observed in a variety of other gram-negative organisms in a survey (55). Although some of these organisms may encounter enterobactin in the gut, the significance of enterobactin utilization to their survival in the host or in the environment is not known for most of these organisms.

It is believed that acquisition of iron is important for virulence, and in some bacterial pathogens, loss of specific iron transport systems correlates with reduced virulence in animal models. However, for many organisms, including *V. cholerae*, it has been difficult to determine the role of specific iron transport systems in virulence. A general requirement for high-affinity iron transport systems in virulence can be inferred from results of virulence studies using *tonB* mutants. *V. cholerae* has two TonB systems (44), which are partially redundant in their transport functions (57). Strains carrying mutations in either of these *tonB* systems have reduced colonization of infant mice as observed in a competition assay, and a strain carrying mutations in both *tonB* systems has an even greater reduction in its competitive index (57). These data suggest that high-affinity iron transport is needed for optimal colonization in this assay; however, no single iron source has been identified as being required for virulence. Strains defective in vibriobactin biosynthesis (59), vibriobactin transport (30), or heme transport (30, 40, 64) are only weakly affected for virulence.

The *irgA* mutant stain MBG40 is more attenuated for virulence than any of the strains carrying mutations in a single iron transport gene (64). However, lack of an identified function for IrgA has made it difficult to understand how it might contribute to the virulence of *V. cholerae*. This has generated debate as to whether IrgA is the receptor for an important, but unidentified, iron source or whether it might have a function unrelated to iron acquisition. In this work we show that IrgA is an outer membrane receptor for enterobactin. The *irgA* single mutants tested in this study used enterobactin as efficiently as their wild-type parent in vitro, presumably due to the presence

of the other enterobactin receptor gene, *vctA*. However, even if IrgA is needed for efficient enterobactin utilization in vivo, it seems unlikely that reduced enterobactin utilization would severely attenuate virulence. *V. cholerae* colonizes the lower portions of the small intestine (3, 4), while *E. coli* is present primarily in the large intestine. Thus, enterobactin is probably not available in significant quantities to *V. cholerae* during colonization of its host. *V. cholerae* may encounter enterobactin as it is shed through the large intestine, but this would not contribute to the competitive index in the infant mouse model.

To resolve this question, we constructed a different *irgA* mutation in O395, the parental strain of MBG40. This *irgA* mutant strain, ARM516, was not significantly defective in its ability to compete with the wild-type parental strain O395 in an infant mouse model (Table 5), suggesting that it is able to colonize and grow at wild-type levels within its host. In addition, the LD_{50} of ARM516 in an infant mouse model was similar to that of the wild-type strain O395. In contrast, MBG40 did not efficiently compete with the parental strain, consistent with the previous characterization of this mutant. The ability of MBG40 to compete with the parental strain was not restored when *irgA* was supplied on a plasmid, providing further evidence that the virulence defect in this strain is not due to loss of IrgA function. From these data we conclude that the virulence defect of MBG40 is specific to that strain. The virulence defect in MBG40 is unlikely to be due to polarity of the Tn*phoA* insertion, because a previous study demonstrated that the gene downstream of *irgA*, which encodes erythrose-4-phosphate dehydrogenase, is not required for virulence (13). It is possible that this defect is the result of toxicity of the *irgA*::*phoA* fusion, although this is unlikely since MBG40 grows normally in vitro in low-iron media in which expression of the fusion protein is maximally induced (21). Alternatively, MBG40 may contain a mutation in addition to the *irgA*::Tn*phoA* insertion. It was shown by Southern hybridization that MBG40 contains only a single Tn*phoA* insertion, (24), implying that this mutation is one not detected by that method. The nature of the colonization defect in MBG40 is not known, but it is of interest to identify the defect, since it appears to have a significant effect on colonization and virulence.

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