INFECTION AND IMMUNITY, May 1996, p. 1756–1761 Vol. 64, No. 5
0019-9567/96/$04.00+0

Relative Importance of Three Iron-Regulated Outer Membrane Proteins for In Vivo Growth of *Vibrio cholerae*

KAREN T. TASHIMA,[1]† PATRICIA A. CARROLL,[1] MARC B. ROGERS,[1] AND STEPHEN B. CALDERWOOD[1,2]*

Infectious Disease Unit, Massachusetts General Hospital, Boston, Massachusetts 02114,[1] and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115[2]

Received 10 November 1995/Returned for modification 3 January 1996/Accepted 23 February 1996

Iron is an essential nutrient to support the growth of most bacterial species. However, iron is not easily available to microorganisms infecting mammalian hosts, because it is largely sequestered by iron-binding proteins, such as transferrin or lactoferrin, or complexed to heme. In response to environmental iron stress, *Vibrio cholerae* produces the siderophore vibriobactin as well as a number of iron-induced outer membrane proteins. Previous data on the role of iron acquisition systems for the intraintestinal growth of mucosal pathogens such as *V. cholerae* are conflicting. In this report, we isolated mutants of *V. cholerae* with Tn*phoA* fusions in each of *viuA*, *hutA*, and *irgA*, as well as strains mutant in each pair of these genes and all three simultaneously, to analyze the role of these iron-induced outer membrane protein receptors for in vivo growth of *V. cholerae*. The fusion between *hutA* and Tn*phoA* in a single copy on the chromosome allowed the study of in vitro regulation of *hutA* in response to iron, *fur*, and *irgB*; transcription of *hutA* was tightly iron regulated (70-fold) and dependent on a functional Fur but did not require IrgB. To investigate the effects of mutations in these iron-induced outer membrane proteins on in vivo growth, we inoculated ileal loops in a rabbit model of infection. This avoids exposure of organisms to the potential killing effects of gastric acid, allows several logarithmic increases in growth in the in vivo environment, and facilitates direct comparison of multiple strains in the same animal to avoid any differences between animals. We grew each mutant to be tested in competition with the wild-type strain in the same loop, to provide an internal control. We confirmed that the inocula for these experiments were grown under conditions of iron stress prior to in vivo inoculation, by measuring the alkaline phosphatase activity of the iron-regulated fusion in each strain. The results confirmed that mutation of *irgA* produced a much more substantial in vivo growth defect than mutation of either *hutA* or *viuA* alone. Double mutants of *irgA* with either *viuA* or *hutA*, or the strain mutant in all three genes, showed an in vivo growth defect comparable to the strain mutant in *irgA* only, suggesting that mutation of *irgA* was the most relevant for in vivo growth. The strain mutant in both *hutA* and *viuA* was also markedly impaired for in vivo growth, suggesting that mutation of both of these iron uptake systems simultaneously can also produce a substantial in vivo growth defect.

Iron is an essential element to support the growth of most bacterial species. However, iron is not freely available to microorganisms infecting mammalian hosts, because extracellular iron is largely sequestered by human iron-binding proteins, such as transferrin in serum and lactoferrin on mucosal surfaces, or complexed to heme. This limitation of the availability of free iron for infecting microorganisms has been termed nutritional immunity. Most bacterial pathogens have developed efficient methods for mobilizing sufficient iron within the mammalian host to support bacterial growth (4). Many bacteria produce siderophores, low-molecular-weight iron chelators, which are able to attract iron away from the host iron-binding proteins transferrin and lactoferrin and which are subsequently transported into the cell as the ferric-siderophore complex through specific uptake mechanisms. Other bacteria utilize the host iron-binding compounds directly as sources of iron and have specific cell surface receptors for transferrin, lactoferrin, or heme.

The bacterial genes involved in iron uptake are regulated by the environmental concentration of iron through the regulatory protein Fur, which represses transcription of these genes in response to sufficient environmental iron (4). In addition to genes involved in iron uptake, bacteria regulate the expression of several genes involved in virulence according to the environmental iron concentration, with enhanced expression of these genes when iron is limiting (21). Examples of virulence genes preferentially expressed under low-iron conditions include Shiga toxin from *Shigella dysenteriae* 1 and diphtheria toxin from *Corynebacterium diphtheriae.*

Vibrio cholerae, the causative agent of the diarrheal disease cholera, produces and secretes a siderophore in response to environmental iron stress (26). This siderophore has been characterized structurally and named vibriobactin (12). We have previously characterized a 74-kDa iron-regulated outer membrane protein of *V. cholerae* (ViuA) that functions as the ferric vibriobactin receptor (33); transcription of this gene is repressed by Fur in the presence of sufficient iron (2).

In addition to ViuA, *V. cholerae* expresses several other outer membrane proteins in response to iron stress (31). One of these proteins, IrgA, is the major 77-kDa iron-regulated outer membrane protein of *V. cholerae*, and we have shown previously that inactivation of the *irgA* gene results in a virulence defect, as well as impaired in vivo growth in the infant mouse (9). Expression of *irgA* is negatively regulated at the transcriptional level by the Fur protein of *V. cholerae* (7, 19) and positively regulated by a transcriptional activator, IrgB,

* Corresponding author. Mailing address: Infectious Disease Unit, Massachusetts General Hospital, Boston, MA 02114. Phone: (617) 726-3811. Fax: (617) 726-7416. Electronic mail address: calderwoods@a1.mgh.harvard.edu.

† Present address: Miriam Hospital, Providence, RI 02906.

which is transcribed divergently from *irgA* (8). IrgA shows significant homology to the TonB-dependent outer membrane proteins of other bacteria, including proteins involved in iron uptake, but IrgA has not been shown to be involved in the utilization of specific iron sources by *V. cholerae* (6).

In addition to the vibriobactin system, *V. cholerae* can also utilize heme or hemoglobin as a sole iron source for growth in iron-restricted conditions (34). Henderson and Payne have recently characterized the heme uptake system of *V. cholerae* and shown that it involves at least a 77-kDa outer membrane protein (HutA) and a 26-kDa inner membrane protein (HutB) (14, 15).

Expression of several bacterial genes in response to iron limitation in vivo has been well documented for systemic infections. For example, Griffiths and Humphreys have shown that *Escherichia coli* expresses the siderophore enterobactin while growing in the peritoneal cavities of infected guinea pigs (10). Normal human serum contains antibodies against enterobactin, as well as the enterobactin receptor of *E. coli*, FepA (11, 25). Gram-negative bacteria directly isolated from patients with urinary tract infections express iron-regulated proteins in purified outer membranes (30).

Expression of iron-regulated genes by bacteria during gastrointestinal infection is less clear. Sciortino and Finkelstein (29) compared outer membrane proteins purified from *V. cholerae* grown in vivo, in the intestines of infant rabbits, with outer membrane proteins purified from *V. cholerae* grown in vitro, in both low- and high-iron conditions. In vivo-grown *V. cholerae* expressed outer membrane proteins that were similar to those seen following iron stress in vitro. However, Jonson et al. performed similar experiments and concluded that in vivo-expressed envelope proteins of *V. cholerae* differed from the envelope proteins seen following in vitro growth in low-iron conditions (17). Henderson and Payne have suggested that mutations in the heme uptake and/or vibriobactin systems of *V. cholerae* impair growth in the intestine of an animal model, suggesting that these systems are necessary for in vivo growth (16). Camilli et al. used in vivo expression technology to examine iron-regulated gene expression in the gut in vivo; they constructed a transcriptional fusion between the *irgA* promoter and the gene for transposase and monitored expression of the *irgA* promoter by scoring resolution of a linked tetracycline resistance gene that was flanked by *res* sites as targets for the transposase (3). They concluded that the *irgA* promoter is not expressed in the intestines of laboratory animals but is expressed in the mouse peritoneal cavity (3). The *irgA*-transposase fusion used by Camilli et al. required iron loading during preparation of the inoculum to prevent in vitro resolution prior to inoculation into the animal; it is possible that the bacteria maintained preexisting iron stores during in vivo growth, preventing an accurate estimate of available iron within the gut.

Although expression of iron-regulated genes and iron uptake systems has been well documented for systemic bacterial infections, the role of iron acquisition systems in the intraintestinal growth of a mucosal pathogen such as *V. cholerae* remains unclear. In the present study, we report the construction of *V. cholerae* strains relevant to studying this question and the use of a rabbit ileal loop model to study the requirement for three iron-regulated outer membrane proteins of *V. cholerae* for in vivo growth.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are shown in Table 1. Vector plasmids pBR322 and pBR328 were from laboratory stock.

Media. All strains were maintained at −70°C in Luria-Bertani (LB) medium containing 15% glycerol. LB medium contained 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter. LB medium with or without the addition of the iron chelator 2,2-dipyridyl (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 0.2 mM was used for determination of alkaline phosphatase activities in high- and low-iron conditions, respectively. Ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDA; Sigma Chemical Co.), deferrated by the method of Rogers (27), was used at a concentration of 1 mg/ml to detect specific growth augmentation of *V. cholerae* in response to 10 μM hemin (Sigma Chemical Co.) or 3 mM ferrous sulfate (Amresco, Solon, Ohio). Syncase broth was also used to produce iron-restricted growth, as previously described (6). Ampicillin (25 or 100 μg/ml), chloramphenicol (12.5 μg/ml), kanamycin (45 μg/ml), streptomycin (100 μ/ml), tetracycline (2 μg/ml), or 5-bromo-4-chloro-3-indolyl phosphate (Amresco; 40 μg/ml) were added as appropriate.

Tn*phoA* mutagenesis of *V. cholerae*. KTT1 and KTT11 were constructed from *V. cholerae* 0395 by Tn*phoA* mutagenesis as previously described (9). Tn*phoA*-containing colonies were screened for the $PhoA^+$ phenotype, indicating fusion of the *phoA* sequences to genes encoding membrane or secreted proteins, on LB agar containing streptomycin, kanamycin, gentamicin, glucose, and 5-bromo-4-chloro-3-indolyl phosphate. $PhoA^+$ strains were screened for negative regulation by iron by determining alkaline phosphatase activities after growth in LB medium with and without dipyridyl, as described previously (9).

Genetic methods. Isolation of plasmid and bacterial chromosomal DNA, restriction enzyme digestions, agarose gel electrophoresis, and Southern hybridization of DNA separated by electrophoresis were performed according to standard molecular biologic techniques (28). DNA restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, Klenow fragment, and calf intestinal alkaline phosphatase were used according to the manufacturers' specifications. Restriction enzyme-digested DNA fragments were separated on 1 to 2% agarose gels; appropriately sized fragments were cut from the gel under UV illumination and purified by the freeze-squeeze technique (36) or by DNA purification resin (Wizard PCR Preps; Promega, Madison, Wis.) after separation on low-melting-temperature agarose gels. DNA fragments used as probes in Southern hybridizations were radiolabelled with [α-^{32}P]dCTP by using a random priming labelling kit (Prime-It II Random Primer Labelling Kit; Stratagene, La Jolla, Calif.). GeneScreen Plus hybridization transfer membranes (DuPont Biotechnology Systems, NEN Research Products, Boston, Mass.) were used according to the manufacturer's protocols for Southern hybridizations.

Plasmids were transformed into *E. coli* strains by standard techniques or were electroporated into *V. cholerae* or *E. coli* strains with a Gene Pulser (Bio-Rad Laboratories, Hercules, Calif.) and modified for electroporation into *V. cholerae* as previously described (8). Electroporation conditions were 2,500 V at 25-μF capacitance, producing time constants of 4.7 to 4.9 ms. All transformants were confirmed by electrophoresis of restriction enzyme digests of plasmid minipreparations.

Strain and plasmid constructions. Inactivation of *V. cholerae fur* and *irgB* in strain KTT1 was carried out as described previously (8, 20). Briefly, SM10λ*pir* (pMBG111) or SM10λ*pir*(pCML13) was conjugated with KTT1; this was followed by double selection for ampicillin and streptomycin resistance. Doubly resistant colonies arise by homologous recombination between the internal fragments of *fur* or *irgB* on the suicide plasmids and the corresponding chromosomal genes on the recipient, causing insertional inactivation. The genotypes of the resulting strains were confirmed by Southern hybridization (not shown) and named KTT139 and KTT141. Strains MBG40, MBG14, and KTT1 contained Tn*phoA* insertions in *irgA*, *viuA*, and *hutA*, respectively.

KTT1 was used as the background strain in which to make additional mutations in *irgA* (PAC24), *viuA* (PAC26), or both (PAC27). Strains were constructed as follows: the chloramphenicol resistance gene of pBR328 was cloned into the *Hpa*I site in *irgA* in pSAB25 (1) to create pPAC16. A 3.7-kbp *Sac*I-*Sph*I fragment from pPAC16 was subcloned into pCVD442, to construct the suicide vector pPAC19, which was propagated in SY327λ*pir* and used with sucrose selection to replace the *irgA* allele in KTT1 by marker exchange, as described previously (20). The genotype of the resulting strain, PAC24, was confirmed by Southern hybridization (not shown). Similarly, the tetracycline resistance gene from pBR322 was cloned into an internal deletion of *viuA* created in pJRB15 (2) to yield pPAC17. A 4.2-kbp *Pvu*II fragment from pPAC17 was subcloned into pCVD442 to construct pPAC20, which was used for marker exchange with *viuA* in KTT1, to yield strain PAC26. PAC27 was derived from PAC24, using pPAC20 for marker exchange with *viuA*, to yield triple mutations in *irgA*, *viuA*, and *hutA*. Strain MR30 was constructed from MBG14 by using marker exchange with pSAB14 (1) to introduce a 1,050-bp internal deletion within *irgA*.

Animal studies. Fifty percent lethal dose (LD_{50}) assays were performed by oral inoculation of 4- to 6-day old CD-1 mice with various doses of bacteria grown in LB medium at 30°C, pelleted, washed, and resuspended in LB medium. Four mice were used per dose of bacteria. Survival was determined at 36 h, and results were analyzed as described previously (9).

A 2.5- to 3.0-kg New Zealand White male rabbit was used for growth and competition of *V. cholerae* strains in ileal loops. The rabbit was fasted overnight and anesthetized with intramuscular ketamine, xylazine, and acepromazine. Prior to skin incision, additional ketamine and xylazine were given, and the rabbit was closely monitored throughout the procedure for redosing with anesthetic to maintain adequate sedation. At the end of the procedure, buprenorphine was

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference(s) or source
V. cholerae		
0395	Classical strain; Sm^r derivative	23
KTT1	0395 *hutA*::Tn*phoA* Sm^r Km^r	This study
KTT139	KTT1 with pMBG111 integrated into *irgB*; Ap^r Sm^r Km^r	This study
KTT141	KTT1 with pCML13 integrated into *fur*; Ap^r Sm^r Km^r	This study
KTT11	0395 *hutA*::Tn*phoA* Sm^r Km^r	This study
MBG13	0395 *hutA*::Tn*phoA* Sm^r Km^r	9
MBG14	0395 *viuA*::Tn*phoA* Sm^r Km^r	2, 33
MBG17	0395 *hutA*::Tn*phoA* Sm^r Km^r	9
MBG40	0395 *irgA*::Tn*phoA* Sm^r Km^r	9
PAC24	0395 *hutA*::Tn*phoA irgA*::Cm^r Sm^r Km^r Cm^r	This study
PAC26	0395 *hutA*::Tn*phoA viuA*::Tc^r Sm^r Km^r Tc^r	This study
PAC27	0395 *hutA*::Tn*phoA irgA*::Cm^r *viuA*::Tc^r Sm^r Km^r Cm^r Tc^r	This study
MR30	0395 *viuA*::Tn*phoA* Δ*irgA* Sm^r Km^r	This study
E. coli		
DH5α	F^- *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)*U169* (ϕ80d *lacZ* Δ*M15*)	13
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir*R6K	24
SY327λ*pir*	Δ(*lac pro*) *nalA recA56 araD argE*(Am) λ*pir*R6K	24
Plasmids		
pCVD442	Suicide vector composed of the *mob*, *ori*, and *bla* regions from pGP704 and the *sacB* gene of *Bacillus subtilis*; Ap^r	5
pHUT3	3-kbp *Hin*dIII-*Sal*I fragment of *hutA* cloned into pAT153; Ap^r	14
pSAB14	pCVD442 with *irgA* containing an internal 1,050-bp deletion; Ap^r	1
pSAB25	pUC19 with 3-kbp *Sma*I-*Mlu*I fragment of *irgA*; Ap^r	1
pPAC16	pSAB25 with 952-bp *Sfu*I fragment from pBR328 containing Cm^r cloned into the *Hpa*I site in *irgA*	This study
pPAC19	pCVD442 with 3.7-kbp *Sac*I-*Sph*I fragment of pPAC16 containing *irgA*::Cm^r; Ap^r Cm^r	This study
pJRB15	pUC18 with 2.7-kbp *Hin*dIII-*Sac*I fragment encompassing *viuA*; Ap^r	2
pPAC17	1.6-kbp *Ssp*I-*Msc*I fragment of pBR322 containing Tc^r, ligated into internal deletion of *viuA* between the *Hpa*I sites in plasmid pJRB15; Ap^r Tc^r	This study
pPAC20	pCVD442 with 4.2-kbp *Pvu*II fragment of pPAC17 containing *viuA*::Tc^r; Ap^r Tc^r	This study
pMBG111	pGP704 with 676-bp internal *Hin*cII-*Bgl*II fragment of *irgB*; Ap^r	8
pCML13	pGP704 with 450-bp *Xba*I-*Sal*I internal fragment of *fur*; Ap^r	20

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

administered for pain control. The abdominal cavity was exposed in a sterile operating room, the small intestine was mobilized out of the abdomen, and 5 ml of phosphate-buffered saline (PBS) was injected intraluminally and used to wash excess bowel contents into the large intestine. A portion of the PBS was withdrawn by syringe and plated on streptomycin-containing LB agar to verify that the intestine contained no streptomycin-resistant normal flora. Ligatures were introduced to divide the intestine into approximately 3- to 5-cm-long segments, each separated from another by a 2- to 3-cm spacer loop; care was taken to avoid ligating mesenteric vessels. The inocula were prepared by growing strains overnight twice in syncase broth at 30°C, and the alkaline phosphatase activities of the inocula were measured to ensure iron limitation. The numbers of organisms per milliliter for the different strains were determined prior to inoculation, by plating serial dilutions on LB plates containing appropriate antibiotics. To determine in vivo growth of each mutant in competition with 0395, duplicate ileal loops were inoculated with approximately equal numbers (10^5 CFU) of 0395 and the mutant strain to be compared (50 μl of 10^{-3} dilutions of the overnight cultures of each). The intestine was returned to the abdominal cavity, and the peritoneum and skin were closed with a continuous suture. Twenty-one hours later, the intestinal loops were reexposed (all had significant fluid accumulation), fluid was taken from each loop, and colony counts were determined for the wild-type and mutant strains by plating serial dilutions on plates containing appropriate antibiotics. The competitive index is defined as the ratio of mutant organisms to 0395 after in vivo growth in the same loop, divided by the ratio of the two organisms at the time of inoculation. A competitive index of less than one denotes an in vivo growth defect of the mutant relative to the wild type.

RESULTS AND DISCUSSION

Tn*phoA* insertions in iron-regulated genes of *V. cholerae* that produce a defect in the utilization of heme iron. After random insertion of Tn*phoA* into the chromosome of *V. cholerae* 0395, colonies were screened for the $PhoA^+$ phenotype by blue color on a plate containing 5-bromo-4-chloro-3-indolyl phosphate. Alkaline phosphatase assays were performed on 77 active Tn*phoA* fusion strains under low- and high-iron conditions, yielding 17 strains with increased alkaline phosphatase activity in low- compared with high-iron conditions. These fusion-containing strains, as well as a set of previously described iron-regulated Tn*phoA* fusions, were assayed for their ability to grow in LB media with EDDA and 10 μM hemin as the sole iron source. Four Tn*phoA* mutant strains showed a mild defect for growth in hemin compared with the wild-type strain 0395 but grew normally when supplemented with $FeSO_4$ (Table 2). The growth defect in hemin for these four strains is milder than

TABLE 2. Growth of *V. cholerae* and various mutants in media containing hemin as the sole iron source

Strain	OD_{600} after overnight growth in LB medium with EDDA[a]		
	Alone	And 10 μM hemin	And 3 mM $FeSO_4$
0395	0.04 (0.03–0.06)	1.64 (1.25–1.91)	2.92 (2.83–3.00)
KTT1	0.07 (0.05–0.09)	0.82 (0.70–0.99)	3.91 (3.17–4.66)
KTT11	0.06 (0.05–0.06)	0.71 (0.60–0.89)	3.40 (2.90–3.90)
MBG13	0.06 (0.06–0.06)	0.79 (0.67–0.94)	3.49 (3.00–3.98)
MBG17	0.06 (0.06–0.06)	0.73 (0.64–0.90)	3.33 (2.95–3.70)
0395(pHUT3)	0.01 (0.0–0.02)	1.66 (1.65–1.67)	
KTT1(pHUT3)	0.02 (0.02–0.03)	1.30 (1.29–1.32)	
KTT11(pHUT3)	0.02 (0.02–0.02)	1.62 (1.61–1.63)	

[a] OD_{600}, optical density at 600 nm. The values are the averages (ranges) from two or three separate experiments. EDDA, 1 mg/ml.

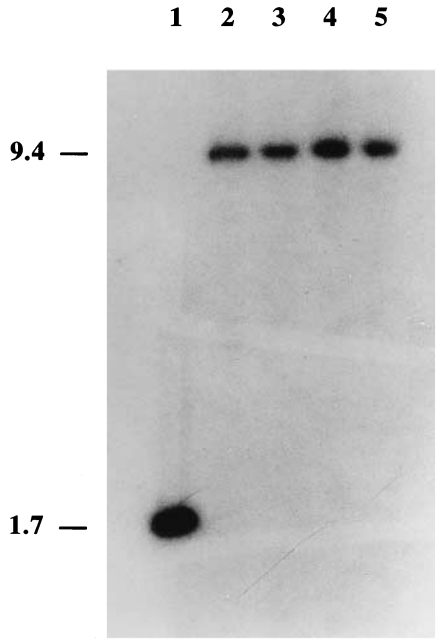

FIG. 1. Southern blot analysis of genomic DNA digested with *Eco*RV and probed with a 1.7-kbp *Eco*RV fragment of pHUT3. Lanes: 1, 0395; 2, KTT1; 3, KTT11; 4, MBG13; 5, MBG17. Sizes on the left are in kilobase pairs.

that reported for the heme uptake mutants of Henderson and Payne, because the latter strains were also deficient for the production of vibriobactin (14).

All four Tn*phoA* fusion strains were shown to have a single Tn*phoA* insertion by Southern hybridization (data not shown). With a probe specific to the previously described *hutA* gene (14), all four strains were shown to have Tn*phoA* insertions in *hutA* (Fig. 1). With the restriction map of *hutA* previously reported by Henderson and Payne (14), the Tn*phoA* insertion in strain KTT1 was shown to be the most proximal in *hutA* by Southern hybridization (data not shown), and this strain was used for further studies. The cloned *hutA* gene on plasmid pHUT3 complemented the growth defects of KTT1 and KTT11 with hemin as a sole iron source (Table 2), confirming that the Tn*phoA* fusions in these strains are in the outer membrane receptor (HutA) for heme uptake.

Regulation of expression of *hutA*. Isolation of a Tn*phoA* fusion in the chromosomal *hutA* gene provided the opportunity to study *hutA* regulation in a single copy. Henderson and Payne have previously utilized a *hutA*::Tn*5 lac* fusion on a multicopy plasmid to show approximately sevenfold regulation of *hutA* in response to iron; this regulation was reduced to some extent in *V. cholerae* in the presence of a *fur* mutation (15).

Using the single-copy, chromosomal Tn*phoA* fusion in strain KTT1 as a recorder activity, we showed that transcription of *hutA* in *V. cholerae* is regulated 70-fold in response to iron (Table 3). Repression by iron was reduced dramatically when the chromosomal *fur* gene was mutated in KTT1; residual two- to threefold regulation by iron despite a *fur* mutation has been seen for other iron-regulated genes in *V. cholerae* (22). Mutation of *irgB*, the positive transcriptional activator of *irgA*, had little effect on expression of *hutA* (Table 3).

Role of iron-regulated outer membrane proteins for virulence and in vivo growth of *V. cholerae*. We have previously shown that mutation of *irgA* in *V. cholerae* produces a defect in virulence, as well as in vivo growth, in infant mice (9). On the other hand, Sigel et al. have previously shown that an inability to produce or transport vibriobactin by *V. cholerae* produces no defect for virulence or for in vivo growth in the same animal model (32). We determined the LD_{50} of the *hutA* mutant KTT1 in infant mice and showed that it had no defect in virulence compared with the wild-type parent (data not shown).

To test for effects on in vivo growth, we decided to use a rabbit ileal loop model and competition for growth with the wild-type strain. The ileal loop model avoids exposure to gastric acid during passage through the stomach and potential differences in susceptibility to acid killing between strains, allows four to five logarithms of in vivo growth for inoculated strains, and facilitates testing multiple strains simultaneously in the same animal, avoiding any strain-to-strain differences between animals. Competition with the wild-type strain serves as an internal control in each loop. We utilized streptomycin-resistant derivatives of *V. cholerae* in our experiments and plated ileal loop contents on streptomycin-containing agar to avoid counting normal flora in the recovered counts; a control experiment showed that the rabbit used contained no streptomycin-resistant normal flora. We grew the inocula under conditions of iron stress prior to in vivo inoculation and incorporated an iron-regulated alkaline phosphatase gene fusion into each mutant so that we could verify the presence of iron stress during growth of the inoculum, by alkaline phosphatase assay.

We utilized three strains containing Tn*phoA* fusions in each of the genes *irgA* (MBG40), *viuA* (MBG14), and *hutA* (KTT1), as well as strains containing mutations in each pair of these genes and all three genes simultaneously. Construction of the mutants was as detailed in Materials and Methods, and the genotype of each mutant was confirmed by Southern hybridization (data not shown). Sequential passage of each strain in vitro in syncase medium for 2 nights was used to prepare an iron-stressed inoculum; alkaline phosphatase assays after growth in syncase medium, compared with growth in LB broth, confirmed that the bacterial inocula were iron stressed (Table 4).

After injection of the inocula and overnight in vivo growth in ligated ileal loops, we compared colony counts of each mutant with those of the wild-type strain grown in the same loop. As shown in Table 5, the results confirmed that mutation of *irgA* produced a much more substantial in vivo growth defect (competitive index, 0.29) than mutation of either *hutA* or *viuA* alone (competitive indices, 0.62 to 0.64). Double mutants of *irgA* with

TABLE 3. Alkaline phosphatase activities of KTT1 and various mutants of KTT1 following growth in high- and low-iron conditions

Strain	Alkaline phosphatase activity (U/OD_{600})[a]		
	High iron	Low iron	Induction ratio
KTT1	20 (16–27)	1,403 (1,176–1,638)	70
KTT141 (*fur*::pCML13)	577 (467–676)	1,373 (784–1,749)	2
KTT139 (*irgB*::pMBG111)	8 (4–14)	946 (927–964)	118

[a] OD_{600}, optical density at 600 nm. The values are the averages (ranges) from two to three separate experiments.

TABLE 4. Growth and alkaline phosphatase activities of various strains grown in high- and low-iron conditions

Strain	OD_{600}[a] in:		Alkaline phosphatase activity (U/OD_{600})[b] in:	
	LB medium	Syncase medium	LB medium	Syncase medium
0395	2.70	1.78	<1	<1
KTT1	2.50	1.72	39 (35–42)	1,507 (1,345–1,669)
MBG14	2.51	2.36	5 (4–5)	139 (138–139)
MBG40	2.64	1.92	4 (4, 4)	247 (240–254)
PAC24	2.62	1.80	32 (29–35)	1,762 (1,548–1,976)
PAC26	2.18	1.57	65 (61–68)	1,466 (1,428–1,503)
MR30	2.57	1.95	16 (14–17)	137 (125–148)
PAC27	2.56	2.35	8 (8, 8)	1,458 (1,445–1,470)

[a] OD_{600}, optical density at 600 nm after overnight growth.
[b] The values are the averages (ranges) from two separate experiments.

either *viuA* or *hutA*, or the strain mutant in all three genes, showed in vivo competitive indices comparable to that of the strain mutant in *irgA* only, suggesting that mutation of *irgA* was the most relevant for in vivo growth. The strain mutant in both *hutA* and *viuA* was also markedly impaired for in vivo growth (competitive index, 0.25), suggesting that mutation of both of these iron uptake systems simultaneously can also produce a substantial in vivo growth defect even without a mutation in *irgA*.

Results of our experiments differ somewhat from previous observations. Sigel et al. showed that strains of *V. cholerae* mutant in the synthesis or uptake of vibriobactin had normal in vivo multiplication following oral infection of an infant mouse at a dose of 5×10^6 organisms; the bacteria in these experiments, however, were not iron stressed prior to inoculation (32). Henderson and Payne subsequently studied fluid accumulation and in vivo growth following inoculation of infant mice with a wild-type strain of *V. cholerae*, as well as isogenic mutants defective in vibriobactin synthesis, heme uptake, or both; they used iron-stressed inocula for their experiments (16). At an inoculated dose of 10^7 organisms, there was no difference in the amount of fluid accumulation in the mice between the four strains. The individual mutants in vibriobactin synthesis or in heme uptake showed slightly diminished in vivo growth, but the double mutant in vibriobactin synthesis and heme uptake showed a dramatic reduction in the number of recovered organisms. When the inoculum size was reduced to 10^6 organisms, all three mutants failed to cause fluid accumulation and all showed a reduction in recovered bacteria relative to the wild-type strain. Of note, however, in both these experiments the number of mutant bacteria recovered following in vivo infection was actually less than the number of bacteria inoculated, suggesting that these experiments measured in vivo survival rather than in vivo multiplication.

TABLE 5. In vivo competition assays between 0395 (wild type) and various mutants

Mutant strain	Competitive index[a]
KTT1 (*hutA*)	0.62 (0.32–0.92)
MBG14 (*viuA*)	0.64 (0.62–0.66)
MBG40 (*irgA*)	0.29 (0.26–0.32)
PAC24 (*hutA irgA*)	0.22 (0.19–0.24)
PAC26 (*hutA viuA*)	0.24 (0.12–0.36)
MR30 (*viuA irgA*)	0.25 (0.20–0.29)
PAC27 (*hutA viuA irgA*)	0.22 (0.20–0.24)

[a] The values are the averages (ranges) of two separate ileal loops, corrected for the input ratio.

We utilized a model that allows four to five logarithms of in vivo growth, competition with the wild-type strain, and direct inoculation of ileal loops to bypass gastric acid, in case the previous observations on survival of mutants represent differential acid susceptibilities rather than differences in in vivo multiplication. Our data suggest that IrgA affects in vivo multiplication in the rabbit small intestine more than the outer membrane receptors involved in vibriobactin or heme uptake. This suggests either that iron acquisition is not needed in this portion of the rabbit gastrointestinal tract for in vivo growth or that sources of iron other than vibriobactin or heme are more relevant for in vivo growth. Kammler et al. have described a system in *E. coli* for the uptake of ferrous iron, encoded by the genes *feoA* and *feoB* (18); mutants of *E. coli* in these genes are impaired in their ability to colonize the mouse intestine (35). In a study by Henderson and Payne (16), a strain of *V. cholerae* mutant in *tonB*, which was impaired in multiple iron uptake systems, was more defective for in vivo growth than the strain doubly mutant for vibriobactin synthesis and heme uptake, suggesting that TonB-dependent iron uptake mechanisms in addition to those for vibriobactin and heme are important for in vivo growth. IrgA is homologous to the *E. coli* TonB-dependent outer membrane receptor proteins, including those involved in iron uptake, but no iron source transported by IrgA has yet been identified. The present study confirms our previous observations that a mutation in *irgA* impairs the in vivo growth of *V. cholerae* in the gastrointestinal tract of an animal model, suggesting that this protein product is necessary for the uptake of an essential nutrient in vivo.

ACKNOWLEDGMENTS

This work was supported by a Public Health Service grant (AI34968) from the National Institute of Allergy and Infectious Diseases to S.B.C. K.T.T. is the recipient of a National Research Service Award from the National Institute of Allergy and Infectious Diseases.

We thank Shelley Payne for providing pHUT3, as well as helpful advice.

REFERENCES

1. **Butterton, J. R., S. A. Boyko, and S. B. Calderwood.** 1993. Use of the *Vibrio cholerae irgA* gene as a locus for insertion and expression of heterologous antigens in cholera vaccine strains. Vaccine **11:**1327–1335.
2. **Butterton, J. R., J. A. Stoebner, S. M. Payne, and S. B. Calderwood.** 1992. Cloning, sequencing, and transcriptional regulation of *viuA*, the gene encoding the ferric vibriobactin receptor of *Vibrio cholerae*. J. Bacteriol. **174:**3729–3738.
3. **Camilli, A., D. T. Beattie, and J. J. Mekalanos.** 1994. Use of genetic recombination as a reporter of gene expression. Proc. Natl. Acad. Sci. USA **91:** 2634–2638.
4. **Crosa, J. H.** 1989. Genetics and molecular biology of siderophore-mediated iron transport in bacteria. Microbiol. Rev. **53:**517–530.
5. **Donnenberg, M. S., and J. B. Kaper.** 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect. Immun. **59:**4310–4317.
6. **Goldberg, M. B., S. A. Boyko, J. R. Butterton, J. A. Stoebner, S. M. Payne, and S. B. Calderwood.** 1992. Characterization of a *Vibrio cholerae* virulence factor homologous to the family of TonB-dependent proteins. Mol. Microbiol. **6:**2407–2418.
7. **Goldberg, M. B., S. A. Boyko, and S. B. Calderwood.** 1990. Transcriptional regulation by iron of a *Vibrio cholerae* virulence gene and homology of the gene to the *Escherichia coli* Fur system. J. Bacteriol. **172:**6863–6870.
8. **Goldberg, M. B., S. A. Boyko, and S. B. Calderwood.** 1991. Positive transcriptional regulation of an iron-regulated virulence gene in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **88:**1125–1129.
9. **Goldberg, M. B., V. J. DiRita, and S. B. Calderwood.** 1990. Identification of an iron-regulated virulence determinant in *Vibrio cholerae*, using Tn*phoA* mutagenesis. Infect. Immun. **58:**55–60.
10. **Griffiths, E., and J. Humphreys.** 1980. Isolation of enterochelin from the peritoneal washings of guinea pigs lethally infected with *Escherichia coli*. Infect. Immun. **28:**286–289.

11. **Griffiths, E., P. Stevenson, R. Thorpe, and H. Chart.** 1985. Naturally occurring antibodies in human sera that react with the iron-regulated outer membrane proteins of *Escherichia coli*. Infect. Immun. **47:**808–813.
12. **Griffiths, G. L., S. P. Sigel, S. M. Payne, and J. B. Neilands.** 1984. Vibriobactin, a siderophore from *Vibrio cholerae*. J. Biol. Chem. **259:**383–385.
13. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
14. **Henderson, D. P., and S. M. Payne.** 1993. Cloning and characterization of the *Vibrio cholerae* genes encoding the utilization of iron from haemin and haemoglobin. Mol. Microbiol. **7:**461–469.
15. **Henderson, D. P., and S. M. Payne.** 1994. Characterization of the *Vibrio cholerae* outer membrane heme transport protein HutA: sequence of the gene, regulation of expression, and homology to the family of TonB-dependent proteins. J. Bacteriol. **176:**3269–3277.
16. **Henderson, D. P., and S. M. Payne.** 1994. *Vibrio cholerae* iron transport systems: roles of heme and siderophore iron transport in virulence and identification of a gene associated with multiple iron transport systems. Infect. Immun. **62:**5120–5125.
17. **Jonson, G., A. M. Svennerholm, and J. Holmgren.** 1989. *Vibrio cholerae* expresses cell surface antigens during intestinal infection which are not expressed during in vitro culture. Infect. Immun. **57:**1809–1815.
18. **Kammler, M., C. Schon, and K. Hantke.** 1993. Characterization of the ferrous iron uptake system of *Escherichia coli*. J. Bacteriol. **175:**6212–6219.
19. **Litwin, C. M., S. A. Boyko, and S. B. Calderwood.** 1992. Cloning, sequencing, and transcriptional regulation of the *Vibrio cholerae fur* gene. J. Bacteriol. **174:**1897–1903.
20. **Litwin, C. M., and S. B. Calderwood.** 1993. Cloning and genetic analysis of the *Vibrio vulnificus fur* gene and construction of a *fur* mutant by in vivo marker exchange. J. Bacteriol. **175:**706–715.
21. **Litwin, C. M., and S. B. Calderwood.** 1993. Role of iron in regulation of virulence genes. Clin. Microbiol. Rev. **6:**137–149.
22. **Litwin, C. M., and S. B. Calderwood.** 1994. Analysis of the complexity of gene regulation by Fur in *Vibrio cholerae*. J. Bacteriol. **176:**240–248.
23. **Mekalanos, J. J., D. J. Swartz, G. D. Pearson, N. Harford, F. Groyne, and M. de Wilde.** 1983. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature (London) **306:**551–557.
24. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
25. **Moore, D. G., R. J. Yancey, C. E. Lankford, and C. F. Earhart.** 1980. Bacteriostatic enterochelin-specific immunoglobulin from normal human serum. Infect. Immun. **27:**418–423.
26. **Payne, S. M., and R. A. Finkelstein.** 1978. Siderophore production by *Vibrio cholerae*. Infect. Immun. **20:**310–311.
27. **Rogers, H. J.** 1973. Iron-binding catechols and virulence in *Escherichia coli*. Infect. Immun. **7:**445–456.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Sciortino, C. V., and R. A. Finkelstein.** 1983. *Vibrio cholerae* expresses iron-regulated outer membrane proteins in vivo. Infect. Immun. **42:**990–996.
30. **Shand, G. H., H. Anwar, J. K. Kadurugamuwa, M. R. W. Brown, S. H. Silverman, and J. Melling.** 1985. In vivo evidence that bacteria in urinary tract infection grow under iron-restricted conditions. Infect. Immun. **48:**35–39.
31. **Sigel, S. P., and S. M. Payne.** 1982. Effect of iron limitation on growth, siderophore production, and expression of outer membrane proteins of *Vibrio cholerae*. J. Bacteriol. **150:**148–155.
32. **Sigel, S. P., J. A. Stoebner, and S. M. Payne.** 1985. Iron-vibriobactin transport system is not required for virulence of *Vibrio cholerae*. Infect. Immun. **47:**360–362.
33. **Stoebner, J. A., J. R. Butterton, S. B. Calderwood, and S. M. Payne.** 1992. Identification of the vibriobactin receptor of *Vibrio cholerae*. J. Bacteriol. **174:**3270–3274.
34. **Stoebner, J. A., and S. M. Payne.** 1988. Iron-regulated hemolysin production and utilization of heme and hemoglobin by *Vibrio cholerae*. Infect. Immun. **56:**2891–2895.
35. **Stojiljkovic, I., M. Cobeljic, and K. Hantke.** 1993. *Escherichia coli* K-12 ferrous iron uptake mutants are impaired in their ability to colonize the mouse intestine. FEMS Microbiol. Lett. **108:**111–116.
36. **Tautz, D., and M. Renz.** 1983. An optimized freeze-squeeze method for the recovery of DNA fragments. Anal. Biochem. **132:**14–19.

Editor: A. O'Brien