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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

DOUGLAS P. HENDERSON AND SHELLEY M. PAYNE*

Department of Microbiology, University of Texas at Austin, Austin, Texas 78712

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***Vibrio cholerae* iron transport mutants were tested for their ability to cause disease in an infant mouse model. The mice were challenged with either the wild-type strain, a vibriobactin synthesis mutant, a heme utilization mutant, or double mutants containing both the vibriobactin synthesis defect and the heme utilization defect. When mice were challenged with 10^7 bacteria, the ability of the double mutant to survive in the intestines was greatly reduced and that of the heme utilization mutant was slightly reduced compared with that of the wild type or the vibriobactin synthesis mutant. When the inoculum size was reduced 10-fold, all of the iron transport mutants failed to colonize the intestines and failed to cause diarrhea in the mice, whereas the wild-type strain was not cleared and elicited a diarrheal response. These data indicate that disruption of either the heme utilization or the vibriobactin uptake system reduces the ability of *V. cholerae* to cause disease. One of the heme utilization mutants, DHH1, was found to be defective also in utilization of vibriobactin and ferrichrome, mimicking the *Escherichia coli* TonB$^-$ phenotype. This mutant was the least virulent of the iron transport mutants tested. Transformation of DHH1 with the recombinant plasmid pHUT4 restored the abilities to use hemin, vibriobactin, and ferrichrome as iron sources, suggesting that pHUT4 encodes a gene(s) involved globally in the iron transport systems. Hybridization of *Vibrio* DNA with the *V. cholerae* heme utilization genes demonstrated the presence of DNA homologous to the genes encoding the outer membrane protein HutA and the inner membrane protein HutB in all the *V. cholerae* strains tested. The probe containing *hutA*, but not that containing *hutB*, also hybridized to DNA from *Vibrio parahaemolyticus*.**

Pathogenic bacteria require iron for growth, but its limited availability in the host makes the element difficult to obtain. Although the human body contains 4 to 5 g of iron, approximately 75% is located intracellularly as hemoglobin (2). Much of the remainder is stored in the liver as ferritin. Trace amounts of iron are complexed to the high-affinity iron-binding protein transferrin, which is present in blood, or lactoferrin, which is present in phagocytes and is secreted onto mucosal surfaces (26).

To survive in the host, bacteria use high-affinity iron transport systems. A number of bacterial species synthesize and secrete siderophores, low-molecular-weight compounds which bind iron and transport it into the cell (18). Some siderophores can remove iron from transferrin, thus competing with this host protein for iron (3, 12). Other bacteria directly utilize host iron complexes as sources of iron. These include the pathogenic *Neisseria* spp. which utilize transferrin (17) or lactoferrin (16) as iron sources and pathogens such as *Haemophilus influenzae* (20) and the pathogenic *Neisseria* spp. (17, 28) which utilize hemoglobin and heme as iron sources.

In several bacterial species, the ability to acquire iron from the host has been shown to correlate with virulence. Williams (27) found a relationship between the virulence of a bacteremic strain of *Escherichia coli* and production of the siderophore aerobactin. Similarly, a virulent strain of the fish pathogen *Vibrio anguillarum* exhibited reduced virulence when cured of a plasmid encoding genes for the biosynthesis of the siderophore anguibactin (3). In both cases, virulence was restored to the siderophore mutants when iron was added to the inoculum. There exists a similar correlation between the ability of pathogenic *Neisseria* spp. to utilize transferrin as an iron source and their ability to cause disease (17, 23).

Vibrio cholerae, the intestinal pathogen that causes the disease cholera, has several iron transport systems. Under low-iron conditions, the bacterium synthesizes and secretes a siderophore called vibriobactin (6). Sigel et al. (24) examined the role of vibriobactin-mediated iron transport in promoting virulence. Both vibriobactin synthesis and vibriobactin transport mutants of *V. cholerae* caused disease in infant mice, suggesting that other mechanisms of iron acquisition function within the host. It was shown subsequently that *V. cholerae* can acquire iron from heme or hemoglobin in a siderophore-independent fashion (25). Under low-iron conditions, *V. cholerae* produces a hemolysin which can lyse erythrocytes and other eukaryotic cells, releasing heme-containing complexes (25). It is possible that heme-containing compounds liberated when intestinal epithelial cells are lysed by the hemolysin may serve as a source of iron, allowing *V. cholerae* to establish an infection.

The heme and hemoglobin iron utilization system of *V. cholerae* was recently characterized in our laboratory (8). Two separate recombinant plasmids, each containing a unique fragment of *V. cholerae* DNA, are required to reconstitute the heme utilization system in *E. coli*, indicating that at least two genes are associated with heme utilization (8). One of the fragments encodes a 26-kDa inner membrane protein (HutB), and the other encodes a 77-kDa outer membrane protein

* Corresponding author. Phone: (512) 471-9258. Electronic mail address: payne@utxvm.cc.utexas.edu.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant phenotype	Source or reference
V. cholerae		
CA401	Wild-type classical strain	5
CA40130	Vibriobactin synthesis (*vib*) mutant of CA401	6
DHH1	Siderophore transport (*sit*) heme utilization (*hut*) mutant of CA40130	8
DHH8	Kan^r *hut* mutant of CA40130 obtained by marker exchange with pHUT156	This study
DHH9	Kan^r *hut* mutant of CA401 obtained by marker exchange with pHUT156	This study
2076-79	Non-O1 strain	J. Oliver
Lou15	El Tor strain	J. Wells
0395	Str^r classical strain	15
E. coli MM294(pRK2013)	Kan^r mobilizing plasmid for triparental matings	R. Meyer
V. vulnificus 324		P. Baumann
V. parahaemolyticus 474801	Clinical isolate	Texas Department of Health
pHUT1	Tet^r 30-kb *Sau*3A1 fragment of CA401 DNA cloned into pLAFR3	8
pHUT3	Amp^r, 3-kb *Hin*dIII-*Sal*I fragment of CA401 DNA cloned into pBR322; encodes 77-kDa outer membrane protein (HutA)	8
pHUT4	Tet^r pLAFR3 containing the 10-kb *Hin*dIII fragment from pHUT1 encoding *hutB*	8
pHUT6	Kan^r, 5.2-kb *Sal*I-*Hin*dIII fragment of pHUT10 cloned into pJRD215	This study
pHUT10	Cm^r pACYC184 containing the same insert as in pHUT4	8
pHUT14	Kan^r, 4.0-kb *Sal*I-*Eco*RI fragment of pHUT10 cloned into pJRD215	This study
pHUT156	pHUT1 with Tn*5* insertion (insertion 1) into 6.0-kb *Eco*RI fragment encoding the 26-kDa inner membrane protein (HutB)	8

(HutA) which is iron regulated at the transcriptional level. These plasmids permit the entire heme molecule to be transported into the cell.

The goal of the present study was to determine the role of the heme iron utilization system in virulence. In this study, isogenic heme utilization mutants of *V. cholerae* were constructed and tested for their ability to cause disease in infant mice. In addition, various *V. cholerae* strains and *Vibrio* species were tested to determine whether genes homologous to those of the heme utilization system of *V. cholerae* CA401 were present.

MATERIALS AND METHODS

Strains. Bacterial strains and plasmids and their sources are listed in Table 1, and some plasmids are depicted in Fig. 1. The recombinant plasmids pHUT10 and pHUT4 are identical inserts cloned into different vectors. The mobilizable vector pLAFR3 was used for constructs which were to be transferred into *V. cholerae*(pHUT4), while pACYC184 was the vector used for maintaining the fragment in *E. coli* for isolation of fragments for hybridization (pHUT10). The broad-host-range vector pJRD215 (4) was used to construct pHUT6 and pHUT14.

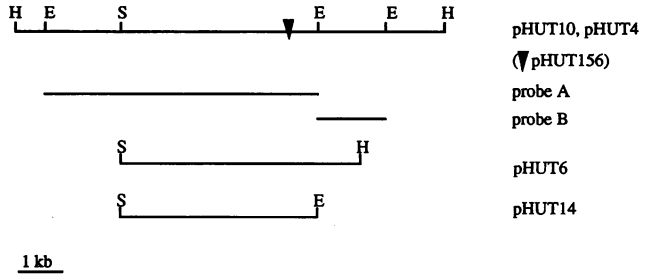

FIG. 1. Restriction map of pHUT10 (pHUT4) and subclones pHUT6 and pHUT14. Restriction enzyme sites *Eco*RI (E), *Hin*dIII (H), and *Sal*I (S), fragments used for Southern hybridization (probes A and B), and the site of the Tn*5* insertion in pHUT156 (arrowhead) are indicated. The *Hin*dIII site in pHUT6 was not present in pHUT10 and was inserted for subcloning.

Media, chemicals, and enzymes. All strains were maintained at −80°C in Luria broth with 20% glycerol. Routine culturing of bacterial strains was done at 37°C in L broth or on L agar. Deferrated ethylenediamine-di-(*o*-hydroxyphenyl acetic acid) (EDDA) (22) was added to L broth or L agar to chelate iron. The following antibiotics (Sigma Chemical Co., St. Louis, Mo.) were used at the indicated concentrations (in micrograms per milliliter): kanamycin, 50; tetracycline, 4.2; and gentamicin, 30. Ferrichrome was a gift from Paul J. Szaniszlo, Department of Microbiology, University of Texas. Restriction enzymes were purchased from New England Biolabs (Beverly, Mass.) and used as described by the supplier.

Construction of mutants. DHH8 and DHH9 were generated from CA40130 and CA401, respectively, by marker exchange with pHUT156, which contains a Tn*5* insertion into a 6-kb *Eco*RI fragment that abolishes heme utilization (insertion 1 in reference 8). pHUT156 and the incompatible plasmid pH1JI were sequentially transferred to CA401 and CA40130 by triparental mating with the mobilizing strain MM294(pRK2013). CA401(pHUT156, pH1JI) and CA40130(pHUT156, pH1JI) were passaged twice in L broth with gentamicin (the marker encoded on pH1JI) and plated on L agar with kanamycin. Colonies were screened for tetracycline sensitivity, which indicated loss of pHUT156, and for the inability to grow on L agar containing hemin and 150 μg of EDDA per ml, which indicated that the Tn*5* mutation on the plasmid had recombined into the chromosome.

Growth assays. Growth assays to test the ability of *V. cholerae* to utilize various compounds as iron sources were performed as follows. Mid-log-phase L-broth cultures were seeded into L agar containing 75 μg of EDDA per ml at 1×10^4 (CA401 and DHH9), 1×10^5 (CA40130 and DHH8), or 5×10^5 (DHH1) bacteria per ml. Five microliters of 20 μM hemin or ferrichrome or 5 μl of an overnight culture of a vibriobactin-producing strain was spotted onto the plate. A sterile disk spotted with 20 μl of 10 mM $FeSO_4$ also was placed

on the plate. The zones of growth around the spots and the disk were measured after 18 to 24 h.

Southern hybridization. Southern hybridization was performed as described by Maniatis et al. (13) with GeneScreen (NEN Research Products, Boston, Mass.). Chromosomal DNA was isolated by the method of Marmur (14). Probes were generated from the 3-kb *Hin*dIII-*Sal*I fragment of pHUT3 which encodes the 77-kDa iron-regulated outer membrane protein (HutA) and from the 6- and 1.5-kb *Eco*RI fragments from pHUT10. pHUT10 encodes the 26-kDa inner membrane protein (HutB) associated with heme utilization (8). The fragments were labeled with [^{32}P]dCTP as described by Koch et al. (11) by using a nick translation system (GIBCO BRL, Gaithersburg, Md.). Hybridizations and washes were performed under stringent conditions for *V. cholerae* DNA and low-stringency conditions for DNA of other species.

Virulence testing of *V. cholerae* mutants. The virulence of CA401, CA40130, and the heme utilization mutants was tested by the fluid accumulation assay of Baselski et al. (1). Four- to six-day-old CFW or BALB/c mice (3 to 10 mice per bacterial strain) were orally inoculated with 10^6 or 10^7 cells. The inoculum was prepared from bacteria grown to mid-log phase in L broth containing 5 to 10 μg of EDDA per ml, which induced sufficient iron starvation to reduce, but not stop, the growth of the culture. Cells were resuspended to the appropriate concentration in T medium (24) containing 0.2% glucose, 0.01% Evans blue, and, where appropriate, 50 μg of kanamycin per ml. After 16 h, the infant mice were sacrificed by cervical dislocation and the stomachs and intestines were removed and weighed. The fluid accumulation ratio was determined as follows: (weight of stomach plus intestines)/(total body weight − weight of stomach plus intestines). After the stomach and intestines had been weighed, the intestines were homogenized in 1 ml of 0.89% saline, and dilutions were plated to determine viable counts. Duplicate serial dilutions from mice infected with DHH8 and DHH9 were plated on L agar with and without kanamycin to ensure that the strains had not reverted to the original phenotype. Statistical analyses of viable counts recovered from the mice were performed with Student's *t* test.

RESULTS

Generation of *V. cholerae* heme utilization mutants. Isogenic heme utilization mutants of wild-type *V. cholerae* CA401 and of its siderophore-defective mutant, CA40130, were generated by marker exchange with pHUT156, creating DHH9 and DHH8, respectively. pHUT156 contains a Tn*5* insertion into the 6.0-kb *Eco*RI fragment of pHUT1. This insertion destroys the ability of pHUT1 to complement one class of *V. cholerae* heme utilization mutants (8). To confirm that DHH8 and DHH9 contained Tn*5* insertions into the proper fragment, chromosomal digests of the strains were hybridized with the 6.0-kb *Eco*RI fragment. Tn*5*, which is approximately 5.6 kb in length, contains no *Eco*RI sites. Thus, a Tn*5* insertion would increase the size of an *Eco*RI fragment by 5.6 kb. When *Eco*RI-digested chromosomal preparations from each of the strains were analyzed, the probe hybridized only to the expected 11.5-kb fragment (data not shown). Chromosomal digests were probed also with the internal *Hin*dIII fragment from Tn*5* to determine if there was more than one Tn*5* insertion; the fragment hybridized to a single *Eco*RI fragment of approximately 11.5 kb in each mutant (data not shown).

Testing the ability of the mutants to utilize various compounds as iron sources. To ensure that the gene disruption affected the utilization of hemin only, the mutants and the parental strains were tested for their ability to utilize various compounds as sources of iron. In the assay, the compounds were spotted onto low-iron medium that had been seeded with the various bacterial strains and the zone of growth around the spots was measured. As expected, CA401 and CA40130 could utilize iron from hemin, vibriobactin, and ferrichrome (Table 2). DHH8 and DHH9 utilized all the iron-containing compounds except hemin, indicating that the transposon insertion disrupted only the heme utilization system and not a gene(s) involved in multiple iron transport systems.

TABLE 2. Growth of *V. cholerae* mutants in medium with vibriobactin or ferrichrome as the iron source

Strain	Zone of growth (mm) with[a]:			
	Hemin	Ferri-chrome	DHH9 (Vib$^+$)[b]	$FeSO_4$
CA401 (Vib$^+$ Hut$^+$)[c]	17	25	25	26
CA40130 (Vib$^-$ Hut$^+$)	12	20	24	18
DHH8 (Vib$^-$ Hut$^-$)	0	22	24	20
DHH9 (Vib$^+$ Hut$^-$)	0	25	23	23
DHH1 (Vib$^-$ Sit$^-$ Hut$^-$)	0	0	0	20
DHH1(pHUT1)	11	22	25	10
DHH1(pHUT6)	12	24	24	14
DHH1(pHUT14)	0	0	0	15

[a] Cultures were seeded into L-EDDA agar, and the indicated bacterial culture or compounds were spotted onto the plates. The zone of growth around each spot and the disk was measured at 18 to 24 h.
[b] Vibriobactin-producing strain.
[c] Vib, vibriobactin synthesis; Sit, siderophore transport; Hut, heme uptake.

DHH1, a deletion mutant defective in heme utilization isolated in a previous study (8), was assayed also. This mutant failed to utilize any of the iron-containing compounds except $FeSO_4$ as an iron source (Table 2). When the heme utilization plasmid pHUT1, or its subclone pHUT4, was transferred to DHH1, the abilities to utilize hemin, vibriobactin, and ferrichrome were restored. Because pHUT4 encodes the HutB protein, which had been shown previously to be required for heme transport (8), additional subclones were constructed (Fig. 1) to determine whether complementation of DHH1 by pHUT4 was due to *hutB* or to other sequences contained on pHUT4. Two subclones, pHUT14, which contains the 4.0-kb *Sal*I-*Eco*RI fragment of pHUT4, and pHUT6, which contains sequences beyond the *Eco*RI site, were tested. Minicell analysis of the proteins encoded by these two plasmids indicated that the 26-kDa HutB protein was expressed by both pHUT6 and pHUT14 (10). However, pHUT6 but not pHUT14 complemented DHH1 (Table 2), indicating that the ability of *V. cholerae* to utilize a variety of iron complexes requires sequences within the 1.2-kb *Eco*RI-*Hin*dIII fragment which is present in pHUT6 but not pHUT14. These data suggest that DHH1 contains a mutation in a gene involved globally in several *V. cholerae* iron transport systems, that sequences contained in pHUT1 complement this defect, and that this function is independent of HutB.

Virulence testing of iron transport mutants. To determine the roles of siderophore and/or heme iron transport in iron acquisition in vivo, the isogenic mutants defective in either or both of the systems were tested for the ability to cause disease in infant mice. Four- to six-day-old BALB/c or CFW mice were orally challenged with 10^7 bacteria. Two barometers of virulence were analyzed: the ability of the organism to elicit a diarrheal response, as indicated by a positive fluid accumulation ratio, and the ability of the organism to colonize the intestine, as determined by the counts of viable bacteria recovered from the intestines.

TABLE 3. Multiplication of wild-type and heme utilization mutants of *V. cholerae* in infant mouse intestines with an inoculum of 10^7 organisms[a]

Strain	Fluid accumulation[b]	CFU in intestines at 18 h[c]	Fold decrease in CFU relative to CA401
CA401	+	3.1 (±3.0) × 10^7	
CA40130 (Vib⁻)	+	1.7 (±1.1) × 10^7	1.8
DHH9 (Hut⁻)	+	7.5 (±2.8) × 10^{6d}	4.1
DHH8 (Vib⁻ Hut⁻)	+	1.4 (±0.74) × 10^{5e}	230
DHH1 (Vib⁻ Sit⁻ Hut⁻)	−	4.5 (±4.3) × 10^{4e}	689

[a] Infant mice were inoculated and assayed as indicated.
[b] +, fluid accumulation ratio of >0.85.
[c] Mean number of *V. cholerae* organisms. Standard deviations are shown in parentheses.
[d] $P = 0.02$ compared with CA401.
[e] $P < 0.005$ compared with CA401.

With the exception of DHH1, which failed to produce a diarrheal response, no differences among the strains in their ability to cause diarrhea in the mice when administered at this dose were observed (Table 3). However, there were differences in the ability of the strains to colonize the intestines of the mice. In mice inoculated with mutant CA40130 (Vib⁻) or DHH9 (Hut⁻), bacterial counts were slightly lower than for those inoculated with CA401. In contrast, viable counts for mice challenged with DHH8 (Vib⁻ Hut⁻) and DHH1 (defective in multiple iron transport systems) were dramatically reduced compared with those from mice challenged with CA401.

It was not clear why most of the mutants elicited a diarrheal response in the mice even though they failed to colonize the intestines. It is possible that the large inoculum contributed to this effect, in that the mice were essentially overwhelmed by the amount of cholera toxin generated by the large quantity of bacteria. To test this, the mice were challenged with lower doses of bacteria. When a 10-fold-smaller inoculum was used, only the mice challenged with CA401 had a positive fluid accumulation ratio (Table 4), suggesting that inoculum size had indeed contributed to the high fluid accumulation ratios of the mutants.

The results shown in Table 4 also indicate that with the smaller inoculum, the viable counts for mice challenged with the iron transport mutants were substantially lower than those for mice challenged with CA401. The numbers of bacteria recovered from the intestines of mice challenged with the wild-type strain indicated survival or multiplication. In contrast, the numbers of iron transport mutants never exceeded

TABLE 4. Multiplication of wild type and heme utilization mutants of *V. cholerae* in infant mouse intestines with an inoculum of 10^6 organisms[a]

Strain	Fluid accumulation[b]	CFU in intestines at 18 h[c]	Fold decrease in CFU relative to CA401
CA401	+	4.4 (±4.90) × 10^6	
CA40130 (Vib⁻)	−	1.1 (±0.96) × 10^{4d}	400
DHH9 (Hut⁻)	−	2.4 (±3.20) × 10^{5d}	18
DHH8 (Vib⁻ Hut⁻)	−	1.6 (±3.00) × 10^{4d}	275

[a] Five- to six-day-old BALB/c mice were inoculated and assayed as indicated.
[b] +, fluid accumulation ratio of >0.85.
[c] Mean number of *V. cholerae* organisms. Standard deviations are shown in parentheses.
[d] $P < 0.05$ compared with CA401.

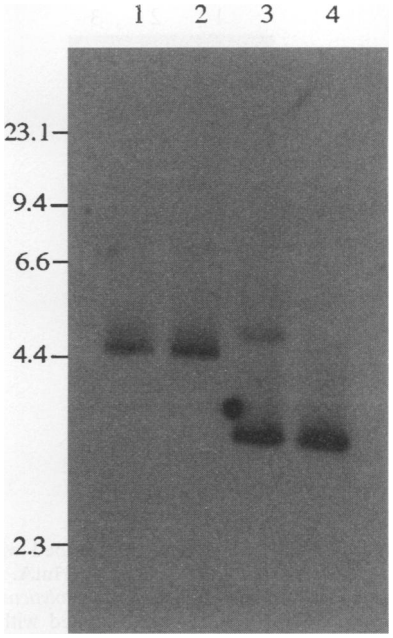

FIG. 2. Southern blot of *V. cholerae* strains probed with the 3.0-kb *Hin*dIII-*Sal*I fragment from pHUT3 encoding HutA. Chromosomal preparations from CA401 (lane 1), 0395 (lane 2), Lou15 (lane 3), and 2076-79 (lane 4) were digested with *Hin*dIII and probed with the 3.0-kb fragment. Sizes of standards (in kilobase pairs) are shown to the left of the autoradiogram.

the original inoculum; viable counts for mice inoculated with the mutants ranged from 18- to 400-fold lower than those from CA401-inoculated mice. The viable counts for mice inoculated with CA40130 were actually lower than those for mice inoculated with either of the marker exchange mutants. This may be influenced by the fact that kanamycin was added to the inocula of DHH8 and DHH9 but not to those of CA401 or CA40130, which have no antibiotic resistance markers. Kanamycin was added to prevent overgrowth of any revertants of the mutants. The presence of the antibiotic may have eliminated the competing flora in the intestines, permitting the marker exchange mutants to survive more readily. This assumption is supported by the fact that non-*V. cholerae* colonies were present on L plates used for viable count determinations for CA40130-infected mice. This phenomenon was not observed on viable count plates for mice infected with the marker exchange mutants (data not shown).

DNA homology of heme utilization genes among *V. cholerae* strains and *Vibrio* species. Because an intact heme transport system was associated with virulence in this model, the prevalence of this transport system in vibrios was determined by Southern hybridization. Growth assays had shown that many *V. cholerae* strains and other *Vibrio* species utilize hemin as a source of iron (7, 19, 25), but it was not known if there is DNA homology among the heme utilization genes in *V. cholerae* strains and other *Vibrio* species. A 3.0-kb *Hin*dIII-*Sal*I fragment containing the entire *hutA* gene (9) was used to probe chromosomal DNA digests. The probe hybridized to 4.5-kb *Hin*dIII fragments in chromosomal preparations from the two classical strains, CA401 and 0395, and to 3.2-kb fragments in chromosomal preparations of the El Tor strain Lou15 and the non-O1 strain 2076-79 (Fig. 2). These data indicate that all the *V. cholerae* strains tested contain DNA homologous to *hutA*.

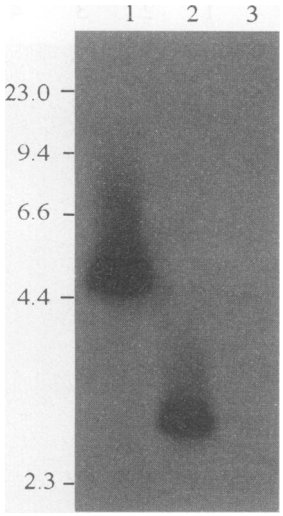

FIG. 3. Southern blot of *Vibrio* species probed with the 3.0-kb *Hin*dIII-*Sal*I fragment from pHUT3 encoding HutA. Chromosomal preparations from CA401 (lane 1), *V. parahaemolyticus* 474801 (lane 2), and *V. vulnificus* 324 (lane 3) were digested with *Hin*dIII and probed with the 3.0-kb fragment. Sizes of standards (in kilobase pairs) are shown to the left of the autoradiogram.

Chromosomal preparations from *Vibrio parahaemolyticus* and *Vibrio vulnificus* were also hybridized to the 3-kb *Hin*dIII-*Sal*I fragment. As shown in Fig. 3, the probe hybridized to a 2.9-kb *Hin*dIII fragment in chromosomal preparations of *V. parahaemolyticus* 474801, while no signal was detected in chromosomal digests of *V. vulnificus*. These data suggest that *V. parahaemolyticus*, but not *V. vulnificus*, contains DNA homologous to *hutA*.

Chromosomal digests from the various strains were also probed with DNA fragments from pHUT10. The gene encoding the inner membrane protein HutB is located within a 6-kb *Eco*RI fragment of pHUT10 (10). Because complementation studies showed that sequences beyond the right-hand *Eco*RI site were required for utilization of multiple iron sources, the 1.5-kb *Eco*RI fragment covering this region was also used for hybridization. *Hin*dIII chromosomal digests of the *V. cholerae* strains were probed with the 6.0-kb (Fig. 1, probe A) or the 1.5-kb (Fig. 1, probe B) *Eco*RI fragment from pHUT10. Both probes hybridized to a 10-kb *Hin*dIII fragment in all the *V. cholerae* strains analyzed (data not shown). These data suggest that the iron transport genes encoded by pHUT10 are present in each of the four strains. The probes did not hybridize to chromosomal digests of *V. parahaemolyticus* 474801 and *V. vulnificus* 324, indicating that neither of these species contains DNA homologous to the heme utilization genes on pHUT10 (data not shown).

DISCUSSION

The ability of pathogens to acquire iron from the host has been linked to virulence. The correlation between iron acquisition and virulence is especially strong among highly invasive bacteria. However, the link between iron transport systems and virulence is less clear among pathogens which infect host surfaces. *V. cholerae* is a surface pathogen that colonizes the intestinal epithelium and secretes a potent toxin that causes profuse watery diarrhea. In this study, iron transport mutants of *V. cholerae* were tested for their ability to cause disease in infant mice. DHH9 contains a heme utilization mutation; DHH8, which contains the same heme utilization defect as DHH9, is also defective in vibriobactin synthesis. CA40130 contains only the vibriobactin synthesis defect. However, it should be noted that CA40130 was generated by chemical mutagenesis, and there exists the possibility that it may contain mutations in addition to the one involved in vibriobactin synthesis. Such mutations, if present, could affect virulence. A fourth mutant, DHH1, is unable to use iron via any of the high-affinity iron transport systems. The results of virulence studies indicate that disruption of either the *V. cholerae* heme utilization or the vibriobactin uptake system reduces the ability of the organism to colonize the intestines and cause disease, particularly when the inoculum size is reduced. The wild-type strain was able to survive in the intestine and in many cases showed net multiplication, whereas the numbers of each mutant recovered were usually significantly lower. When both iron transport systems are disrupted, the ability of the organism to colonize the intestines is reduced even further. Thus, there is a correlation between the ability to acquire iron by using the siderophore system or the heme utilization system and the ability of *V. cholerae* to cause disease. These data suggest that the heme utilization system or the vibriobactin uptake system may serve as a backup when the other system is incapable of transporting the available iron, but maximum virulence requires the expression of both systems.

As had been observed previously (24), the siderophore synthesis mutant CA40130 was found to be capable of causing diarrhea in infant mice when they were challenged with a relatively large inoculum. However, differences in the ability of CA40130 to colonize and multiply in vivo were noted in the two studies. This may reflect the iron status of the cells prior to inoculation. In the previous study, the bacteria were not iron starved before inoculation and may have been able to initiate multiplication by using internal iron stores, whereas in this study the bacteria were slightly iron starved to eliminate internal iron stores prior to inoculation. The difference in the ability of CA40130 to multiply in vivo in the two studies also may have been influenced by the strain of mice used. Only CFW mice were used in the earlier study (24); in the current study, CFW mice were used to confirm the results with the larger CA40130 inoculum. When the same strain of CFW mice was no longer available, BALB/c mice were used for all other experiments. The precise role of the iron status of the bacterial cells and the effect of the strain of mice remain to be determined. Nevertheless, the data obtained with smaller inocula and different growth conditions indicate that the ability of iron transport mutants to colonize the intestines is reduced.

Experiments with mutant DHH1 indicated that this strain has a defect not only in the ability to utilize hemin, but also in the ability to utilize the siderophores vibriobactin and ferrichrome. The phenotype of DHH1 is similar to that of *E. coli tonB* mutants, in that the mutation results in the inability of the mutants to transport several compounds into the cell (21). TonB is an inner membrane protein required for the transport of a number of compounds, such as various siderophores and the vitamin B_{12}, into the cell (21). When pHUT4 was transferred to DHH1, the ability to utilize iron from hemin, vibriobactin, and ferrichrome was restored. Sequences required for complementation mapped outside the region encoding HutB. These data suggest that pHUT4 encodes both a protein required specifically for heme transport (HutB) and a protein(s) involved globally in multiple iron transport systems and which may be the TonB counterpart in *V. cholerae*. It should be mentioned that the iron-regulated outer membrane protein HutA, which is required for heme utilization, shares

homology with a number of TonB-dependent proteins in *E. coli* and other bacteria (9). However, the *V. cholerae* heme iron utilization system is independent of *E. coli* TonB, and none of the recombinant heme utilization plasmids complement a *tonB* defect in *E. coli* (9). Therefore, if pHUT4 encodes a TonB-like protein, the protein is sufficiently different from *E. coli* TonB that the two are not functionally interchangeable. DNA homologous to the sequences encoding this function were found in all the *V. cholerae* strains but not in any of the other *Vibrio* species tested.

The heme iron utilization genes are conserved among the various *V. cholerae* strains. Probes constructed from the two recombinant heme utilization plasmids, pHUT10 and pHUT3, hybridized to chromosomal digests of classical, El Tor, and non-O1 strains, but restriction fragment length polymorphism was observed. The probe hybridized to a 4.5-kb *Hin*dIII fragment in chromosomal digests from the two classical strains and to a 3.2-kb *Hin*dIII fragment from the El Tor and non-O1 strain. Because the 3.0-kb *Hin*dIII fragment is of sufficient size to encode *hutA*, it is possible that the variability among the strains lies outside the *hutA* gene.

Although both *V. parahaemolyticus* and *V. vulnificus* can utilize hemin as an iron source, only *V. parahaemolyticus* contains DNA homologous to that of the *V. cholerae* heme utilization system. The homology exists between the *hutA* gene of *V. cholerae* and a region on the chromosome of *V. parahaemolyticus*. Homology between *V. cholerae* CA401 genes encoded on pHUT10 and *V. parahaemolyticus* was not detected. These data suggest that *V. parahaemolyticus* encodes a HutA counterpart but not a gene homologous to that encoding the inner membrane protein HutB or the region encoding the gene required for multiple iron transport functions. The heme utilization system of *V. vulnificus* appears to be distinct from that of *V. cholerae* CA401.

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