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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

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The regulation of *hutA*, the *Vibrio cholerae* gene encoding a 77-kDa iron-regulated outer membrane protein required for heme iron utilization, was characterized, and the DNA sequence of the gene was determined. A *hutA*::Tn*5 lac* fusion generated previously (D. P. Henderson and S. M. Payne, Mol. Microbiol. 7:461–469, 1993) was transformed into Fur$^-$ and Fur$^+$ strains of *Escherichia coli* and *V. cholerae*. The results of β-galactosidase assays on the transformed strains demonstrated that transcription of *hutA* is regulated by the Fur repressor protein in *E. coli* and at least partially regulated by Fur in *V. cholerae*. Analysis of the DNA sequence of *hutA* indicated that a sequence homologous to the *E. coli* consensus Fur box was present in the promoter region of *hutA*. The amino acid sequence of HutA is homologous to those of several TonB-dependent outer member proteins. However, when the *V. cholerae* heme utilization system, which requires one or more genes encoded by the recombinant plasmid pHUT10 in addition to *hutA* carried on a second vector, was transferred to a wild-type strain and an isogenic *tonB* mutant of *E. coli*, the *tonB* mutant could utilize heme iron as efficiently as the wild-type strain. These data indicate that the *V. cholerae* heme utilization system reconstituted in *E. coli* does not require a functional TonB protein. The *tonB* mutant transformed with the heme utilization plasmids could not utilize the siderophore ferrichrome as an iron source, indicating that none of the genes encoded on the heme utilization plasmids complements the *tonB* defect in *E. coli*. It is possible that a gene(s) encoded by the recombinant heme utilization plasmids encodes a protein serving a TonB-like function in *V. cholerae*. A region in the carboxy terminus of HutA is homologous to the horse hemoglobin ζ chain, and the amino acids involved in forming the heme pocket in the ζ chain are conserved in HutA. These data suggest that this region of HutA is involved in heme binding.

Iron is essential for the growth of most bacteria, but in nature the element is highly insoluble in an aerobic environment and therefore unavailable to most organisms. Inside the human body, most iron is in the cell in the form of hemoglobin or other iron-containing proteins or is stored as ferritin. Trace amounts of iron are found outside the cell complexed to high-affinity iron-binding proteins such as lactoferrin or transferrin (2). The scarcity of free iron inside the body makes it difficult for pathogens to obtain sufficient amounts of the element to survive and multiply in the host.

To acquire iron from their environment, bacteria have evolved specialized systems that permit them to scavenge iron from their environment or to directly utilize host iron-containing proteins as iron sources. For example, many bacteria synthesize and secrete siderophores, low-molecular-weight compounds which bind ferric iron (Fe^{3+}) (33). Certain siderophores bind Fe^{3+} with sufficient affinity to remove the element from host proteins such as transferrin (5, 21). A number of bacteria directly utilize transferrin and lactoferrin (20, 29, 30, 37) or hemoglobin (6, 26, 29, 35, 43) as iron sources.

Many iron transport systems characterized to date involve iron-regulated outer membrane receptors which bind a specific iron-containing compound and facilitate transport of the iron into the cell. The expression of many iron-regulated receptors is controlled at the transcriptional level by an iron-binding repressor protein called Fur (ferric uptake regulation) (19). Under conditions of iron sufficiency, Fur binds to a highly conserved region called the Fur box upstream of iron-regulated genes and blocks transcription (1, 7, 15). Under low-iron conditions, repression by Fur is relieved and the genes are transcribed.

Another common feature of many iron transport systems in *Escherichia coli* and other gram-negative bacteria is their dependence on the TonB inner membrane protein, which provides energy for transport of the ligand across the outer membrane (36). TonB can span the periplasmic space (9) and physically interact with the outer membrane receptor (41) in a highly conserved region called the TonB box (3, 16, 32). This interaction is thought to lead to a conformational change in the receptor protein, permitting transport of the ligand across the outer membrane into the periplasmic space (36).

Vibrio cholerae, the intestinal pathogen that causes the disease cholera, can acquire iron in two ways. Under low-iron conditions, the organism synthesizes and secretes the siderophore vibriobactin (14), which binds ferric iron. Ferric vibriobactin then binds to the vibriobactin receptor, ViuA, a 74-kDa iron-regulated protein (4, 42), to allow transport of iron into the cell. Transcription of *viuA* is controlled by the *V. cholerae* Fur protein (4), which exhibits 76% amino acid homology with the *E. coli* Fur protein (22). *viuA* contains two predicted Fur boxes in its promoter operator region (4). Analysis of the predicted amino acid sequence of ViuA indicates that the protein has limited homology with TonB-dependent outer membrane proteins in *E. coli* and other bacteria (4). It should be mentioned that *V. cholerae* IrgA, an

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TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference or source
V. cholerae		
0395	Str^r, classical strain	28
CML14	Str^r Ap^r *fur* mutant of 0395	S. Calderwood
E. coli		
MFT-5	Tn*5* insertion into *fur*	M. McIntosh
RK4321	Sm^r *entA*	R. Kadner
RK4338	Δ*tonB* mutant of RK4321	R. Kadner
SURE	Tc^r *hsdR supE44 lacZ*ΔM15 F′, host for pBluescript	Stratagene
Plasmids		
pHUT3	Cb^r, 3-kb *Hin*dIII-*Sal*I fragment containing *hutA* cloned into pAT153	17
pHUT10	Cm^r, 10-kb *Hin*dIII fragment containing gene for 26-kDa HutB protein	17
pHUT3::Tn*5 lac*	Tn*5 lac* insertion into *hutA*	17
pABN203	Tc^r, cloned *E. coli fur* gene	J. Neilands

iron-regulated outer membrane protein of unknown function, is homologous to a number of TonB-dependent receptors (11), suggesting that transport systems related to the TonB-dependent systems in *E. coli* are present in *V. cholerae*.

V. cholerae can also acquire iron from heme or hemoglobin in a siderophore-independent fashion (43). The heme and hemoglobin iron utilization system of *V. cholerae* was recently characterized in our laboratory (17). In that study, the two recombinant plasmids pHUT3 and pHUT10, each of which contains a unique fragment of *V. cholerae* DNA, were found to be necessary to reconstitute the heme utilization system in *E. coli*. The study indicated that at least two genes are associated with heme utilization; one of the genes encodes a 26-kDa inner membrane protein (HutB), and the other encodes a 77-kDa outer membrane protein (HutA) which is iron regulated at the transcriptional level. It also was demonstrated that the entire heme molecule is transported into the cell. The goal of the present study was to determine the DNA sequence of *hutA* and the predicted amino acid sequence of the 77-kDa HutA protein, to further characterize the regulation of its expression, and to determine whether the *V. cholerae* heme utilization system requires a functional *E. coli* TonB.

MATERIALS AND METHODS

Strains. Bacterial strains, plasmids, and their sources are listed in Table 1.

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. Ethylenediamine-di-(*o*-hydroxyphenyl acetic acid) (EDDA), deferrated as described by Rogers (38), was added to L broth or L agar to chelate iron.

Transformation of *E. coli* and *V. cholerae*. Recombinant plasmids were transferred to *E. coli* by calcium chloride transformation (25). Plasmids were transferred to *V. cholerae* by electroporation as described previously (17).

β-Galactosidase assays. β-Galactosidase assays were performed on mid-log-phase cultures as described by Miller (31) on strains transformed with pHUT3::Tn*5 lac*. *E. coli* MFT-5 and MFT-5(pABN203) transformed with the *lac* fusion plasmid were grown in L broth or L broth with 500 μg of EDDA per ml; *V. cholerae* 0395 and CML14 transformed with the *lac* fusion plasmid were grown in L broth with 80 μM $FeSO_4$ or L broth with 100 μg of EDDA per ml. Independent experiments were performed on each strain grown under the same conditions on three separate occasions.

Growth assays. Growth assays to test the ability of *E. coli* transformed with pHUT3 and pHUT10 to utilize various compounds as iron sources were performed as follows. Overnight L-broth cultures were seeded into L agar containing 75 μg of EDDA per ml at 10^4 bacteria per ml (*E. coli* RK4321) or 5×10^5 bacteria per ml (other strains). Five-microliter spots each of 80 μM hemin and 500 μM ferrichrome were spotted onto the plate, and a sterile disk saturated with 20 μl of 10 mM $FeSO_4$ was placed on the plate. The zones of growth around the spots and the disk were measured after 18 to 24 h.

DNA sequencing. Plasmids containing DNA inserts to be sequenced were maintained in *E. coli* SURE, and both strands of DNA were sequenced by the dideoxy-chain termination method of Sanger et al. (39), using the Sequenase version 2.0 sequencing kit (United States Biochemical, Cleveland, Ohio) and α-^{35}S-dATP (NEN, Boston, Mass.) as the label. Primers were synthesized by the Department of Zoology at the University of Texas at Austin. Double-stranded sequencing was performed on pHUT3, and/or single-stranded sequencing was performed by using pBluescript containing various fragments of the insert in pHUT3. The reaction mixtures were electrophoresed through 6% acrylamide denaturing gels at 45-W constant power. After electrophoresis, the gels were fixed to remove urea, dried on a slab gel dryer, and autoradiographed. The amino acid sequence of HutA was analyzed by using PROSCAN by DNASTAR*.

Nucleotide sequence accession number. The GenBank/EMBL accession number for the primary nucleotide and amino acid sequence of HutA is L27149.

RESULTS

Fur regulation of *hutA*. Because a number of iron-regulated genes in *E. coli* and *V. cholerae* are regulated by the Fur repressor protein, experiments were performed to determine if the iron regulation of the gene for the 77-kDa heme transport protein (HutA) is also controlled by Fur. A Fur^- strain (MFT-5) and a Fur^+ isogenic strain [MFT-5(pABN203)] of *E. coli* were transformed with pHUT3::Tn*5 lac*, which contains a *lac* fusion to *hutA*. β-Galactosidase assays were then performed on the transformed strains grown under conditions of iron depletion or iron excess. As shown in Fig. 1, β-galactosidase activity in the Fur^- strain transformed with the *hutA-lac* fusion was virtually the same regardless of the iron concentration, indicating that *hutA* was no longer regulated by iron. Iron regulation of the gene was restored when MFT-5(pHUT3::Tn*5 lac*) was transformed with pABN203, which contains a functional *fur* gene. β-Galactosidase activity of the Fur^+ strain of *E.*

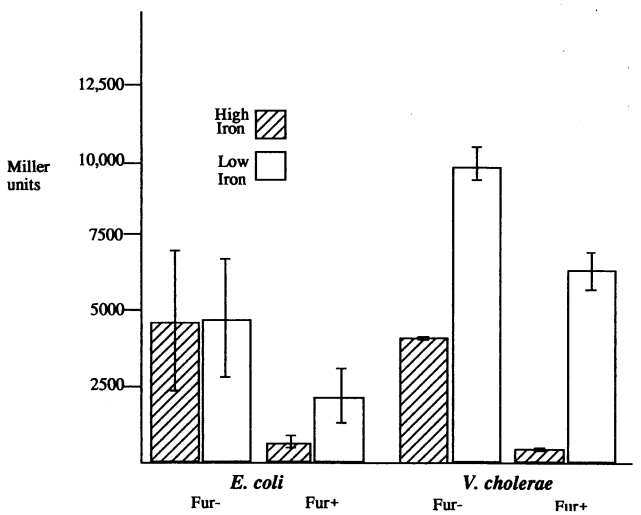

FIG. 1. Effects of iron level on β-galactosidase activities of *E. coli* and *V. cholerae fur* mutants transformed with pHUT3::Tn*5 lac*. Cultures were grown in iron-rich or iron-poor medium. After 4 h of growth, β-galactosidase activity was measured. The Fur$^-$ strain of *E. coli* is MFT-5, and the Fur$^+$ strain is MFT-5 containing pABN203, which encodes a functional *fur* gene. The Fur$^-$ strain of *V. cholerae* is CML14, a derivative of the wild-type Fur$^+$ strain 0395. All four strains were transformed with pHUT3::Tn*5 lac*. The data are averages of three independent experiments performed on different cultures, and the bars indicate the standard deviations.

coli was approximately fourfold higher in low-iron medium than in high-iron medium.

The fusion was also tested for β-galactosidase activity in 0395, a Fur$^+$ strain of *V. cholerae*, and in CML14, an isogenic *fur* mutant of 0395. When assayed in the Fur$^+$ background of the parent strain, β-galactosidase activity was 13-fold higher when the strain was grown in low-iron medium than in high-iron medium. In contrast, β-galactosidase activity in CML14(pHUT3::Tn*5 lac*) was only twice as high when the strain was grown under low-iron conditions than under high-iron conditions (Fig. 1). It is not clear from these data why transcription of *hutA* is somewhat iron regulated in the Fur$^-$ strain of *V. cholerae* but not in the Fur$^-$ strain of *E. coli*. It is possible that in *V. cholerae*, other regulatory elements, in addition to the Fur repressor protein, are involved in the regulation of *hutA*. Nevertheless, the data indicate that transcription of *hutA* is regulated by Fur in *E. coli* and to some extent in *V. cholerae*.

Nucleotide sequence of *hutA*. The nucleotide sequence of *hutA* and its promoter region was determined (Fig. 2). The predicted −35 and −10 regions, Shine-Dalgarno sequence, and terminator region are indicated. A putative Fur box is located immediately downstream of the −35 region and overlaps the −10 region of the gene. The precursor form of HutA, which contains a predicted leader sequence of 21 amino acids, is 693 amino acids in length and has a predicted molecular weight of 77,291. The mature protein has a predicted molecular weight of 75,051 and an isoelectric point of 4.60. The average hydrophobicity of the mature protein is −0.57, indicating that the protein is hydrophilic in nature.

Figure 3 shows a comparison of the predicted Fur box of *hutA* with the *E. coli* consensus Fur box and the predicted Fur boxes from two *V. cholerae* genes. The *hutA* Fur box shares 13 of 19 nucleotides with the consensus sequence of the *E. coli* Fur box. The invariable regions (underlined regions in Fig. 3) in the *E. coli* consensus Fur box, as determined by Griggs and Konisky (15), are identical to similarly located regions in the *hutA* Fur box. The *hutA* Fur box has 12 bases in common with the *irgA* Fur box, 11 bases in common with the *viuA* upstream Fur box, which overlaps the −35 region, and 10 bases in common with the *viuA* downstream Fur box, which overlaps the −10 region. No other regulatory motifs which might explain the residual iron regulation in the *V. cholerae fur* mutant were identified by sequence analysis.

Homology between HutA and TonB-dependent outer membrane proteins. Comparisons of the amino acid sequence of HutA with those of other outer membrane proteins indicated that HutA has significant homology with several iron-regulated, TonB-dependent outer membrane proteins in *E. coli*, including BtuB (vitamin B_{12} receptor; 22.0% identity), Cir (the colicin I receptor; 20.4% identity), FhuE (receptor for coprogen, ferrioxamine B, and rhodotorulic acid; 18.8% identity), and the cloacin DF13/aerobactin receptor protein (18.8% identity). In addition, HutA has 21.0% identity with HemR, the putative heme receptor in *Yersinia enterocolitica*, which also is iron regulated and TonB dependent (44). Lundrigan and Kadner (24) identified several highly conserved regions among TonB-dependent outer membrane proteins in *E. coli*. The regions of greatest homology between HutA and the TonB-dependent outer membrane proteins listed above cluster around these highly conserved regions. Figure 4 shows two of these conserved regions from TonB-dependent outer membrane receptors and a comparison with similarly located regions of HutA.

Figure 4A shows a comparison of an extended region near the amino terminus in HutA and HemR, a TonB-dependent outer membrane protein in *Y. enterocolitica* (44). In the two sequences, each of which spans approximately 200 residues, 60% of the amino acids are conserved or replaced by a functionally similar amino acid. A region of especially high homology is indicated by the horizontal bar. In this region, 32 of 36 amino acid residues are identical or functionally similar. This region of HutA also has a comparable degree of homology with similarly located sequences in BtuB and Cir (data not shown) and in the *V. cholerae* outer membrane proteins ViuA (the vibriobactin receptor) and IrgA, a protein of unknown function (11).

Because many iron transport systems are dependent upon TonB, HutA was examined for the presence of an amino acid sequence which had been shown in other genes to be associated with TonB dependence. A comparison of this region, termed the TonB box, of the *E. coli* vitamin B_{12} receptor BtuB and the putative *Y. enterocolitica* heme receptor HemR with a similarly located and homologous region of HutA is shown in Fig. 4B. The most highly conserved amino acids among the TonB boxes analyzed to date are indicated by boldface letters in the BtuB and HemR sequences. Although there is considerable homology in this region of the three proteins, only one of the three highly conserved amino acid residues is present in the predicted HutA sequence. The highly conserved threonine (the second residue in the BtuB and HemR TonB boxes) is replaced in the HutA sequence by a glutamic acid residue, introducing a charged amino acid at this position. These data indicate that HutA has homology with other TonB-dependent outer membrane proteins but that the purported TonB box of HutA is different from that of known TonB-dependent receptors. This finding suggested that the *V. cholerae* heme transport system is TonB independent or that a TonB analog in *V. cholerae*, if present, recognizes a different sequence.

Role of *E. coli tonB* in heme transport. Experiments were performed to test whether the *V. cholerae* heme utilization

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TATAACCCTTTGATTATAAAATTCATTTAGAAAACTCAAGTCCTGTTCTTTTCACATTAAGCAAACAATTAAAC    74
                                                                      -35

ACAAATGATAGCAATTATCATTAATATTTATTAGAATTCCTGCGTTTTACTCAACATGGAAATTGCAGGTAGTT   148
     Fur Box      -10                                                  SD

CAA ATG TAT AAA AAG TCT CTG CTC TCT AGC GCG ATC ATG CTA GCA CTC GTG CCT TCA   205
    met tyr lys lys ser leu leu ser ser ala ile met leu ala leu val pro ser

GCA TAC GCG GAT GAT TAT GCC TCA TTC GAT GAA GTA GTA GTA TCT ACA ACT CGC TTG   262
ala tyr ala asp asp tyr ala ser phe asp glu val val val ser thr thr arg leu

AAT ACT CAA ATA ACT GAC ACC GCA GCA TCA GTC GCT GTT ATC AAT GCC TCA GAC ATT   319
asn thr gln ile thr asp thr ala ala ser val ala val ile asn ala ser asp leu

GAA CAG CAG ATG GCT GAA GAT ATC GAA GGC CTA TTC AAA TAT ACC CCT GGT GTA ACA   376
glu gln gln met ala glu asp ile glu gly leu phe lys tyr thr pro gly val thr

TTA ACA ACG AAT TCG CGT CAG GGC GTT CAA GGG ATC AAT ATC CGA GGT ATC GAA GGA   433
leu thr thr asn ser arg gln gly val gln gly ile asn ile arg gly ile glu gly

AAC CGC ATC AAG GTT ATC GTT GAC GGT GTA GCT CAA CCC AAC CAG TTT GAC TCC GGA   490
asn arg ile lys val ile val asp gly val ala gln pro asn gln phe asp ser gly

AAT TCA TTT TTA AAC TCA TCT CGA GTT GAT ATC GAT ACA GAT ATG GTG AAA TCG GTT   547
asn ser phe leu asn ser ser arg val asp ile asp thr asp met val lys ser val

GAA ATT GTT AAG GGT GCG GCA TCA TCA CTA CAA GGG TCA GAT GCA ATT GGC GGT ATT   604
glu ile val lys gly ala ala ser ser leu gln gly ser asp ala ile gly gly ile

GTT GCT TTT GAA ACC AAA GAT CCT GCC GAT ATA CTC AAA GGC CGT AAT ATG GGT GGC   661
val ala phe glu thr lys asp pro ala asp ile leu lys gly arg asp met gly gly

TAT GCG AAA CTG AAT TAT TCG TCA TCA GAC AAA ACA TTT AGT GAG TCT ATC GCT TTA   718
tyr ala lys leu asn tyr ser ser ser asp lys thr phe ser glu ser ile ala leu

GCC AAT AAA TCT GGT GAT TTA GAA TCG TTA GTC GCA TAT ACG CGC CGT GAT GGG CAA   775
ala asn lys ser gly asp leu glu ser leu val ala tyr thr arg arg asp gly gln

GAA ATC CAA AAC TTT GGT TCG CCA GAC CAA CAA GAT AAC AAT GCT AAT AAT TTA CTA   832
glu ile gln asn phe gly ser pro asp gln gln asp asn asn ala asn asn leu leu

GTC AAG TTA CAG TAT CAG CTA AAT CCT AAG CAT AGA CTT GAA TTT TCA GGG AAC TAT   889
val lys leu gln tyr gln leu asn pro lys his arg leu glu phe ser gly asn tyr

ATT CGC AAT AAA AAT GAT TTA GAA AAC TTA GAA TTT TCT GGC TAC AAG AAC GCT TCT   946
ile arg asn lys asn asp leu glu asn leu glu phe ser gly tyr lys asn ala ser

GGT ACT GAT GAA ACG ACT CAA TAT CAG TTA GGC ATT AAA CAT ATA TGG GAT GCC GAG  1003
gly thr asp glu thr thr gln tyr gln leu gly ile lys his ile trp asp ala glu

TTT TCT CTT GCT GAT CGT ATA ACA TGG CAG TTT GAT GTC GTA GGT AAA GAA GAG ACT  1060
phe ser leu ala asp arg ile thr trp gln phe asp val val gly lys glu glu thr

GGT ATT ACT GAT CGC ACA AGC AAA TCA AAT GGA AAT ATT CAG AAA AAA GAT TAT TTA  1117
gly ile thr asp arg thr ser lys ser asn gly asn ile gln lys lys asp tyr leu

TAC TCC GAT AAG GGT TTT TCA TTT GAT AGC CAG TTA GAC AAA TCA TTT ATG GTT TCC  1174
tyr ser asp lys gly phe ser phe asp ser gln leu asp lys ser phe met val ser

AAT ACA GAA CAT TAT ATC GTA TAT GGT TTT TCT TTA AGT GAT AAA GAT ATA GAA AAT  1231
asn thr glu his tyr ile val tyr gly phe ser leu ser asp lys asp ile glu asn
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FIG. 2. Nucleotide sequence of *hutA* and the predicted amino acid sequence. The −35 region, the −10 region, and the Shine-Dalgarno sequence (SD) are underlined and labeled. The potential Fur box is shown in boldface letters. A vertical arrowhead marks the predicted signal peptidase cleavage site. The termination codon is marked by three dots, and a region of dyad symmetry downstream of the termination codon is underlined. This region of dyad symmetry is the probable transcription terminator.

system reconstituted in *E. coli* requires a functional TonB protein. *E. coli tonB* deletion mutant RK4338 and its parent strain RK4321 were transformed with pHUT3 and pHUT10, both of which are required to reconstitute the heme utilization system in *E. coli*. The strains were then tested for their ability to utilize hemin as an iron source. It was reasoned that if the *E. coli* TonB protein is required for heme transport, RK4338(pHUT3,pHUT10) would not grow as well as RK4321(pHUT3,pHUT10) when hemin was the sole iron source. As shown in Table 2, both of the transformed strains, but neither of the untransformed strains, grew well on medium containing hemin as the source of iron. Utilization of hemin in the absence of TonB could indicate that the cloned *V. cholerae* DNA included a gene that complements the *E. coli tonB* mutation. Therefore, the strains were assayed for their ability to utilize ferrichrome as an iron source, since this transport system requires a functional TonB protein. The results indicated that only the $TonB^+$ strains could utilize ferrichrome as an iron source, and the ability to utilize ferrichrome was independent of the heme utilization plasmids (Table 2). Thus, the recombinant heme utilization plasmids do not encode a protein that complements the *E. coli tonB* defect, and the *V.*

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ACA AAC CAA GAG TTT AAC TCA ATT GGG AAA AAC AAC GTT ATT TTC TAC ATA CCT AAT 1288
thr asn gln glu phe asn ser ile gly lys asn asn val ile phe tyr ile pro asn

GCA TCT GAA AAA CGT TAT GGC TTT TTT ATC CAA GAT GAA ATT GCA TTC GAT AAC TTA 1345
ala ser glu lys arg tyr gly phe phe ile gln asp glu ile ala phe asp asn leu

ATC GTT ACT CCA GGG ATC CGT TTC GAT TCC TTC GAA ACA AAA CCC GGA GAT ACA AGT 1402
ile val thr pro gly ile arg phe asp ser phe glu thr lys pro gly asp thr ser

GCT AAC CCG AGC CTA AAT GAT GCA AGT GAA TAC AAG AAA TAT TCA GAC TCA GCA TTA 1459
ala asn pro ser leu asn asp ala ser glu tyr lys lys tyr ser asp ser ala leu

ACA GCG AGA CTA GGT ACT GTT TAT AAA TTG AAC CAA GAA AAT CGT CTG TTT GCA CAA 1516
thr ala arg leu gly thr val tyr lys leu asn gln glu asn arg leu phe ala gln

ATT AGC CAA GGT TTC AGA GCA CCA GAC TTC CAG GAG CTG TAT TAC TCT TTT GGT AAT 1573
ile ser gln gly phe arg ala pro asp phe gln glu leu tyr tyr ser phe gly asn

CCA GCT CAT GGA TAT GTT TTT AAA CCA AAC CCT AAT CTA GAA GCA GAA GAC AGT GTT 1630
pro ala his gly tyr val phe lys pro asn pro asn leu glu ala glu asp ser val

TCT TAT GAG CTT GGA TGG CGT TAT AAC GCA GAT AGT GTA AGT AAT GAA CTA TCT ATT 1687
ser tyr glu leu gly trp arg tyr asn ala asp ser val ser asn glu leu ser ile

TTC TAC AGT GAT TAT GAT AAC TTT ATT GAT AGT CAA ATT GTA TCT GGT AGC TTC AAA 1744
phe tyr ser asp tyr asp asn phe ile asp ser gln ile val ser gly ser phe lys

ACA AGG GAT GCT GTA CAC CAA TCA ATT AAT ATT GAT AAA GCA ACA ATT AAA GGG ATT 1801
thr arg asp ala val his gln ser ile asn ile asp lys ala thr ile lys gly ile

GAA CTT TCT AAC CAA TTC TTT TGG GAT AGA TTT ATG CCT ATT GTA GGC TTT AGC TCT 1858
glu leu ser asn gln phe phe trp asp arg phe met pro ile val gly phe ser ser

CGT ATT GCT GCT GCA TAT ACG GAA GGC AAA GAT GGC AAT GGA AAA CCA CTC AAT AGC 1915
arg ile ala ala ala tyr thr glu gly lys asp gly asn gly lys pro leu asn ser

GTA AGT CCT TGG AAT GCT GTC ACT GGT ATC AAT TAT GAT TCA GAA AAT AAT TGG GGT 1972
val ser pro trp asn ala val thr gly ile asn tyr asp ser glu asn asn trp gly

ACT GCA GTT AAT CTG ACT TAT ACC GCG AAG AAA AAA GCT AGT GAG ATC AAT GGT GAC 2029
thr ala val asn leu thr tyr thr ala lys lys lys ala ser glu ile asn gly asp

TAC CAA CCA ATC TCT TCA GCA ACG GTT ATT GAT GTT ACA GCC TAC TAC AAA CCT ATT 2086
tyr gln pro ile ser ser ala thr val ile asp val thr ala tyr tyr lys pro ile

AAA GAT TTA ACA CTA CGT GCA GGT GTG TTC AAT CTT ACA GAT GAA GAA TAT TAT AAC 2143
lys asp leu thr leu arg ala gly val phe asn leu thr asp glu glu tyr tyr asn

TGG AAT GAT GTT CGC GGT TTA CCT AGT GAA GAT AAA GAT AAG ACT CAG GCT AAG CGT 2200
trp asn asp val arg gly leu pro ser glu asp lys asp lys thr gln ala lys arg

AAC TTT GGT ATT ACA GCT AAG TAC GAA TTC TAAATTGTGTGATTCATAGAGATGGTTTCAATTTT 2265
asn phe gly ile thr ala lys tyr glu phe ...

CTTAGCTGAAGATCGAGCTTGTAATTTTGCTCTTGAAACCGCTCTTTCTTGATCTGGTAGTTCTGAGATACCTCT 2340

TCAGACAAAGGAGTTTGATCAGATATCTCA 2370
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FIG. 2—*Continued.*

cholerae heme utilization system reconstituted in *E. coli* does not require a functional *E. coli tonB*. These results do not exclude the possibility that the recombinant plasmids encode a protein that serves a TonB-like function in heme transport but that is not interchangeable with the *E. coli* TonB and therefore does not permit uptake of ferrichrome.

Homology between HutA and the horse hemoglobin ζ chain. Further comparisons of HutA with other proteins indicated that a 151-amino-acid region in the carboxy portion of HutA has 18.5% identity with the horse hemoglobin ζ chain, an embryonic form of hemoglobin supplanted by the α chain early in development. The ζ chain is highly homologous to the α chain, and the regions of the α chain thought to be involved in forming the heme pocket are highly conserved in the ζ chain (8, 10, 34).

Figure 5 shows a comparison of HutA with the horse hemoglobin ζ chain. An area of particularly high homology between HutA and the horse hemoglobin ζ chain spans a 15-amino-acid region of HutA (indicated by the horizontal bar). When this region of HutA is compared with a homologous region of the horse hemoglobin ζ chain, 12 of 15 residues are identical to that of the ζ chain or contain functional substitutions. Sixteen amino acid residues, twelve of which are uncharged amino acids, are involved in forming the heme pocket in the hemoglobin ζ chain (10). These residues are shown in boldface letters in Fig. 5. Twelve of sixteen residues in the heme pocket of the horse ζ chain are conserved or replaced by an amino acid of similar function in HutA (Fig. 5). The proximal histidine residue (raised **H** in Fig. 5) that binds the heme iron in the hemoglobin chain is replaced in HutA by aspartic acid.

A more detailed comparison of the amino acids involved in forming the heme pocket and the corresponding residues in HutA is shown in Table 3. As indicated in Table 3, two of four residues in the ζ chain that are not conserved in HutA are uncharged residues. The corresponding residues in HutA are

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V. cholerae hutA          A C A A A T G A T A G C A A T T A T C
E. coli consensus(13)     G A T A A T G A T A A T C A T T A T C
V. cholerae irgA (12)     G A A A T T A A G A A T A A T T A T C
V. cholerae viuA          G C A A A T G A G A A T G C T T T A C
 (upstream) (11)
V. cholerae viuA          G T G A A T T A T T A A G A T T C T C
 (downstream) (10)
```

FIG. 3. Homology between the *hutA* Fur box and Fur boxes from other iron-regulated genes. The *hutA* Fur box was compared with the *E. coli* Fur box consensus sequence and with the Fur boxes from *V. cholerae irgA* and *viuA* (*viuA* contains two Fur boxes). The invariable regions identified by Griggs and Konisky (15) are underlined in the predicted *hutA* Fur box and in the *E. coli* consensus Fur box. Boldface letters indicate the bases that each Fur box shares with the predicted *hutA* Fur box. The numbers in parentheses indicate the number of common positions that each sequence shares with the *hutA* Fur box.

also uncharged but not functionally similar. These are indicated by the letters in parentheses in Table 3. Because the carboxy region of HutA has homology with the heme pocket of the horse hemoglobin ζ chain, it may play a role in the binding of heme by *V. cholerae.*

DISCUSSION

Many iron transport systems in gram-negative bacteria involve iron-regulated outer membrane receptors. The genes that encode iron-regulated receptors in *E. coli* and *V. cholerae* are controlled at the transcriptional level by the Fur repressor protein. In this study, *hutA*, the *V. cholerae* gene encoding a 77-kDa iron-regulated outer membrane protein, was sequenced and its regulation was characterized.

Fusion of *hutA* sequences to the *lac* reporter gene allowed measurement of *hutA* expression under different conditions. β-Galactosidase assays performed on Fur^- and Fur^+ strains of either *E. coli* or *V. cholerae* transformed with pHUT3::Tn*5 lac* indicate that *hutA* is iron regulated at the transcriptional level in a Fur-dependent manner in both organisms. However, transcription from the *lac* fusion in the Fur^- mutant of *V. cholerae* was still influenced by the iron concentration of the medium, whereas a mutation in *fur* resulted in complete loss of repression in *E. coli*. Litwin and Calderwood (23) also noted residual regulation by iron when measuring the effect of this *fur* mutation on expression of the iron-regulated gene *irgA*. They used the identical *fur* mutation in *V. cholerae* MBG-40, which contains a Tn*phoA* fusion to the iron-regulated gene *irgA*. Expression was threefold higher when the Fur^- strain was grown in low-iron medium versus high-iron medium. These data suggest the existence of a regulatory element(s) in addition to *fur* which influences the expression of iron-regulated genes in *V. cholerae*. Given the extensive homology between

TABLE 2. Effect of an *E. coli tonB* mutation on heme utilization

Strain	Zone of growth (mm)[a]		
	Hemin	Ferrichrome	Iron
RK4321 ($TonB^+$)	0	20	20
RK4338 ($TonB^-$)	0	0	13
RK4321(pHUT3,pHUT10)	12	24	15
RK4338(pHUT3,pHUT10)	18	0	12

[a] Cultures were seeded into L agar containing EDDA, and various iron-containing compounds were spotted onto the medium or onto sterile disks placed on the medium. The zones of growth around the spots or the disks were measured after 18 to 24 h.

A

```
HutA (48)   VAVINASDIEQQMAEDIEGLFKYTPGVTLTTNSRQGVQGINIRGI
                V:V::A     : A     ::::  PG:T:T ::R   Q:: :RG
HemR (64)   VTVVEADTPTSETATSATDMLRNIPGLTVTGSGRVNGQDVTLRGY

HutA        EGNRIKVIVDGVAQPNQFDSGNSFLNSSRVDIDTDMVKSVEIVKG
              : :  :VDG: Q  :  :G:  LNS: :D    :VK VEIV:G
HemR        GKQGVLTLVDGIRQGTD--TGH--LNSTFLD--PALVKRVEIVRG
                    *                                 *  *

HutA        AASSLQGSDAIGGIVAFETKDPADILKGRNMGGYAKLN-YSSSDKT
            ::: L GS:A:GG:::::ET D:AD:L   : :GY   :  :::D :
HemR        PSALLYGSGALGGVISYETVDAADLLLPGQNSGYRVYSAAATGDHS
              **
             (Y)

HutA        FSESIALANKSGDLESLVAYTRRDGQEIQNF-GSPDQQDNNANNL
            F: : :   :::D:::::::  RD  :I::  G    :D:  :N:
HemR        FGLGASAFGRTDDVDGILSFGTRDIGNIRQSDGFNAPNDETISNV

HutA        LVKLQYQLNPKHRLEFSGNYIRN
            L K   :::  : L  :  Y  N
HemR        LAKGTWRIDQIQSLSANLRYYNN
```

B

```
BtuB  (26)    DTLVVTA
HutA  (28)    DEVVVST
HemR  (44)    DTMVVTA
```

FIG. 4. Homology between HutA and TonB-dependent outer membrane proteins. (A) Homology between HutA and the amino-terminus region of the *Y. enterocolitica* HemR protein. A sequence near the amino terminus of the *Y. enterocolitica* HemR protein was compared with a similar region of *V. cholerae* HutA. The numbers in parentheses indicate the position in the unprocessed protein of the first amino acid listed. Conserved amino acids are indicated by capital letters between the compared sequences, and substitutions of functionally similar amino acids are marked by colons. The amino acids marked with an asterisk are those found by Nau and Konisky (32) to be highly conserved among TonB-dependent receptors in *E. coli*. The horizontal bar above a portion of the sequence marks a highly conserved region between the two proteins. (B) Homology between HutA and the TonB boxes of two TonB-dependent receptors. The TonB box region from two TonB-dependent outer membrane proteins was compared with that of the *V. cholerae* HutA protein. The proteins are BtuB and HemR. The numbers in parentheses indicate the position in the unprocessed protein of the first amino acid shown. The most highly conserved residues in the TonB box as described by Nau and Konisky (32) are shown in boldface letters. The underlined residues in the purported HutA TonB box are the amino acids that are different from the invariable residues in the BtuB and HemR TonB boxes.

the *hutA* Fur box and the *E. coli* consensus Fur box and between the *V. cholerae* and *E. coli* Fur proteins (22), it is not surprising that *hutA* is regulated similarly in *E. coli* and *V. cholerae*. The predicted *hutA* Fur box contains 13 of 19 bases that are identical to the *E. coli* consensus sequence, and the two Fur boxes are identical in the invariable regions.

To date, genes from at least four *V. cholerae* systems are known to be regulated by Fur. These include the siderophore synthesis (43) and transport (4) genes, the genes involved in hemolysin production (43), the genes encoding IrgA (12) and IrgB (13), and the gene encoding HutA. That both the genes for hemolysin production and the genes for synthesis of HutA are iron regulated suggests that the two systems may work together to acquire heme iron. The hemolysin may lyse intes-

```
Horse ζ chain   (30) QRLFSSYPQTKT-YFPHFDLH-EG-----SPQLRAH
                      : F : P: :  Y:: F:   :G     :P:L A:
HutA            (461) SQGFRA-PDFQELYYS-FGNPAHGYVFKPNPNLEAE

Horse ζ chain         GSKVAAAVGDAVKSIDNVAGALAKL-SELHAYI-LR
                      :S V:  :G   :: D:V:  L: : S: : :I  :
HutA                  DS-VSYELGWRYNA-DSVSNELSIFYSDYDNFIDSQ

                                                        _
Horse ζ chain         VDPVNFKF---LSHCLLVTLASRLPADFTADAHAAW
                      : : :FK    : : : :  A: :  ::    : W
HutA                  IVSGSFKTRDAVHQSINIDKAT-I-KGIELSNQFFW

                      ____________
Horse ζ chain         DKFLSIV--SSVLTEKYR
                      D:F::IV  SS ::  Y
HutA                  DRFMPIVGFSSRIAAAYT
```

FIG. 5. Homology between HutA and the horse hemoglobin ζ chain. The amino acid sequence of HutA was compared with that of horse hemoglobin ζ chain. Conserved amino acids are indicated by letters between the two sequences, and substitutions of functionally similar amino acids are indicated with colons. Boldface letters in the ζ chain mark amino acids involved in forming the heme pocket. The histidine residues in the ζ chain important in proper functioning or heme binding are indicated by a lowered **H** (distal histidine) or a raised **H** (proximal histidine). The numbers in parentheses indicate the position in the unprocessed protein of the first amino acid listed. The horizontal bar above a portion of the sequence marks a highly conserved region between the two proteins.

tinal epithelial cells, leading to the release of heme and heme-containing proteins which are then utilized as an iron source.

The protein sequence of HutA was deduced from the DNA sequence and analyzed for functional motifs and homology to other proteins. Similarity between HutA and TonB-dependent proteins was noted, particularly in the amino terminus. The homology spans the TonB box region, which is believed to physically interact with TonB, and another amino-terminal region of approximately 200 amino acids. These data suggest that the genes for HutA and the various TonB-dependent receptors may have evolved from the same ancestral genes. Although the amino portion has remained highly conserved, the carboxy portion may have diverged to reflect the specific interaction between the protein and the compound to be transported.

TABLE 3. Comparison of potential heme-binding amino acid residues in HutA with the residues in the horse hemoglobin ζ chain that form the heme pocket

Amino acid position in ζ chain	Helix notation	Amino acid residue	
		Horse ζ chain	HutA[a]
42	C7	Y	Y
43	CD1	F	Y
45	CD3	H	
46	CD4	F	F
58	E7	H	E
61	E10	K	
62	E11	V	V
83	F4	L	F
86	F7	L	(Y)
87	F8	H	D
91	FG3	L	(S)
93	FG5	V	I
97	G4	N	S
98	G5	F	F
101	G8	L	V
139	H19	L	I

[a] Identical or functionally similar amino acids are indicated by letters; uncharged residues replaced with other uncharged but not functionally similar amino acids are indicated by letters in parentheses.

Considering the homology between HutA and other TonB-dependent proteins, it was surprising that the *V. cholerae* heme utilization system did not require a functional *E. coli tonB*. Although a region of HutA has substantial homology with the TonB box from TonB-dependent outer membrane receptors, only one of three invariable amino acid residues is conserved in this region of HutA. It is possible that a TonB-like protein in *V. cholerae* interacts with this region in a manner that is comparable to the interaction between the *E. coli* TonB and the outer membrane receptor. Moreover, the region in HutA that is similar to a TonB box may have diverged sufficiently so that it cannot interact properly with the *E. coli* TonB protein. It would be of interest to replace this region of HutA with the TonB box from an *E. coli* receptor and determine if function is maintained. It should be noted that the *V. cholerae* IrgA, whose function has yet to be determined, is also homologous to various TonB-dependent proteins (11). IrgA contains a region that is almost identical to the TonB box of various TonB-dependent proteins. Unlike the comparable region in HutA, the proposed TonB box in IrgA contains the three highly conserved amino acids found in the invariable region of known TonB boxes (11).

The experiments involving an *E. coli tonB* mutant transformed with the heme utilization plasmids indicate that none of the proteins encoded by the heme utilization plasmids complements the *tonB* mutation in the *E. coli* strain. *V. cholerae* may encode a TonB-like protein, but it must be sufficiently different from the *E. coli* TonB so that the two proteins are not interchangeable. To date, no TonB-like protein has been identified in *V. cholerae*. Our laboratory has isolated a *V. cholerae* mutant which exhibits a phenotype comparable to that of a *tonB* mutant (18). The mutant cannot utilize hemin and the siderophores vibriobactin and ferrichrome as iron sources. When the strain is transformed with pHUT4, a plasmid containing the same insert present in pHUT10, the ability to utilize the aforementioned compounds is restored. These data suggest that pHUT4 and pHUT10 encode a TonB-like protein.

Amino acid sequence analysis of HutA indicates that the carboxy terminus is homologous to the heme-binding pocket of the horse hemoglobin ζ chain. Most of the amino acids involved in forming the heme pocket of the ζ chain are uncharged or hydrophobic residues. Although the overall homology between the ζ chain and the carboxy region of HutA is not high, the amino acids involved in the interaction of the ζ chain with heme are highly conserved in HutA. The overall difference between the ζ chain and the homologous region in HutA may reflect the different roles of the two proteins. Heme is a stable part of the hemoglobin molecule and is critical for the proper function of hemoglobin. Therefore, it is important that heme be tightly complexed to the hemoglobin molecule. Conversely, the interaction between HutA and heme may be transient in nature. HutA needs to bind heme with sufficient affinity to remove it from the environment but not so tightly that heme cannot be released inside the cell.

Because HutA is an iron-regulated outer membrane protein that shares homology with a number of TonB-dependent receptors in *E. coli* and other bacteria and because HutA is required for transport of heme, it seems a likely candidate for a heme receptor. As previously mentioned, several iron trans-

port systems in *E. coli* also have iron-regulated outer membrane proteins which act as receptors that bind specific compounds before they are transported across the outer membrane.

Recently, *hemR*, the gene encoding the putative heme receptor in *Y. enterocolitica*, was sequenced and its regulation was characterized (44). The HemR protein has a number of features in common with the *V. cholerae* HutA protein described in this report. Both are iron-regulated outer membrane proteins of approximately 77 to 78 kDa, and both are involved in uptake of the heme molecule into the cell. Both genes are regulated at the transcriptional level by the Fur repressor protein, and each contains a putative Fur box in the promoter region. Each protein shares significant homology with a number of TonB-dependent outer membrane proteins in *E. coli* and other organisms. However, the heme transport system in *Y. enterocolitica* requires a functional *tonB* which can be provided by the *E. coli tonB*, whereas that of *V. cholerae* is independent of *E. coli tonB*. The carboxy portion of the HutA protein also contains homology to the horse hemoglobin ζ chain. Stojiljkovic and Hantke (44) did not report homology between HemR and any hemoglobin chains. Our comparison of the carboxy portion of HemR with the horse hemoglobin ζ chain indicates that the proteins share only weak homology (data not shown). Thus, it is possible that the mechanism of heme binding is different in the two organisms.

It is not clear if HutA also serves as a receptor for hemoglobin. *V. cholerae* grows well with hemoglobin as the sole source of iron, and mutants defective in heme transport are also unable to utilize hemoglobin. If HutA is the hemoglobin receptor, this would be analogous to the transferrin and lactoferrin utilization systems of the pathogenic *Neisseria* spp., in which the outer membrane receptors bind transferrin and lactoferrin, and the iron is removed and then shuttled into the cell (27, 40). A similar mechanism may be used to obtain heme from hemoglobin. Alternatively, an additional protein may be required to bind hemoglobin and transfer heme to HutA, or a protease may remove the heme from hemoglobin, permitting HutA to bind heme.

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