Characterization of the *Plesiomonas shigelloides* Genes Encoding the Heme Iron Utilization System

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Plesiomonas shigelloides is a gram-negative pathogen which can utilize heme as an iron source. In previous work, *P. shigelloides* genes which permitted heme iron utilization in a laboratory strain of *Escherichia coli* were isolated. In the present study, the cloned *P. shigelloides* sequences were found to encode ten potential heme utilization proteins: HugA, the putative heme receptor; TonB and ExbBD; HugB, the putative periplasmic binding protein; HugCD, the putative inner membrane permease; and the proteins HugW, HugX, and HugZ. Three of the genes, *hugA*, *hugZ*, and *tonB*, contain a Fur box in their putative promoters, indicating that the genes may be iron regulated. When the *P. shigelloides* genes were tested in *E. coli* K-12 or in a heme iron utilization mutant of *P. shigelloides*, *hugA*, the TonB system genes, and *hugW*, *hugX*, or *hugZ* were required for heme iron utilization. When the genes were tested in a *hemA entB* mutant of *E. coli*, *hugWXZ* were not required for utilization of heme as a porphyrin source, but their absence resulted in heme toxicity when the strains were grown in media containing heme as an iron source. *hugA* could replace the *Vibrio cholerae hutA* in a heme iron utilization assay, and *V. cholerae hutA* could complement a *P. shigelloides* heme utilization mutant, suggesting that HugA is the heme receptor. Our analyses of the TonB system of *P. shigelloides* indicated that it could function in *tonB* mutants of both *E. coli* and *V. cholerae* and that it was similar to the *V. cholerae* TonB1 system in the amino acid sequence of the proteins and in the ability of the system to function in high-salt medium.

Plesiomonas shigelloides is a gram-negative bacterium associated with diarrheal disease in humans (4). The organism has been reported to cause several types of gastroenteritis, including acute secretory gastroenteritis (33), an invasive shigellosis-like disease (35), and a cholera-like illness (55). Extraintestinal infections, such as meningitis, bacteremia (2), and pseudoappendicitis (13), are also associated with *P. shigelloides* infection.

Many bacterial pathogens have iron transport systems that play a critical role in allowing the organism to establish an infection (for reviews, see references 31 and 41). Several bacterial pathogens, including *P. shigelloides* (9), obtain iron from heme or heme-containing compounds. Heme iron utilization systems have been examined in many gram-negative pathogens, including *Vibrio cholerae* (20, 21, 38), *Vibrio vulnificus* (30), *Shigella dysenteriae* (37, 60), *Escherichia coli* O157:H7 (54), *Serratia marcescens* (16, 26, 27), yersiniae (22, 50, 51, 53), *Haemophilus* (8, 11, 24, 32), neisseriae (7, 29, 52, 64), *Pseudomonas* (23, 28, 39), and *Porphyromonas gingivalis* (47). Many heme iron utilization systems studied to date require an outer membrane receptor which binds heme from the environment (for a review, see reference 58). TonB, with the aid of ExbBD, is thought to interact with the receptor to allow movement of heme into the periplasm. A periplasmic binding protein then moves the heme across the periplasm, and inner membrane permeases transport the heme into the cytoplasm.

A question yet to be resolved is what happens to the heme once it enters the cytoplasm of the cell. It is clear that heme iron is utilized as an iron source, but it is not clear how the heme is broken down. Recently, genes have been identified in the pathogenic neisseriae (64) and in the gram-positive pathogen *Corynebacterium diphtheriae* (45) that encode heme oxygenases which may break down the heme, releasing the iron into the cell. The heme oxygenase genes are required for heme iron utilization in both organisms. Whether other heme iron-utilizing bacteria use a similar mechanism to remove the iron from heme has not been determined.

In a previous study, *P. shigelloides* heme iron utilization genes were isolated (9). The goal of the present study was to further characterize the *P. shigelloides* system by examining the genes required for heme iron utilization and determining the functions of the proteins they encode. Our results indicate that the heme utilization system of *P. shigelloides* is most similar to that of *V. cholerae* in terms of the amino acid sequence and function of many of the proteins, the regulation of some of the genes, and in the linkage of TonB system genes to the heme iron utilization locus. In addition, we have identified a set of genes, one or more of which is required for heme iron utilization but not utilization of heme as a porphyrin source.

MATERIALS AND METHODS

Strains. Bacterial strains, plasmids, and their sources are listed in Table 1. DPH-2 was a spontaneous heme utilization mutant isolated from *P. shigelloides* 9 after nalidixic acid enrichment as previously described (20). DHE-1, a *hemA entB* mutant of *E. coli*, was created by P1 transduction of a lysate from the *entB* mutant *E. coli* AB1515.24 (48) into the *hemA* mutant RK1065L.

Media, chemicals, and enzymes. Bacterial strains were routinely grown at 37°C in Luria (L) broth or on L agar. Ethylenediamine-di-(*o*-hydroxyphenyl acetic acid) (EDDA), deferrated as described by Rogers (44), was added to L broth or L agar to chelate nonheme iron. Antibiotics and supplements were used in the following concentrations: carbenicillin, 25 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 30 μg/ml (for *E. coli*) and 10 μg/ml (for *P. shigelloides*); tetracycline, 12.5 μg/ml (for *E. coli*) and 4.2 μg/ml (for *P. shigelloides*); δ-aminolevulinic acid (ALA), 80 μg/ml; and hemin, 7.6 μM.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
P. shigelloides		
P. shigelloides 9	Type strain	9
DPH-2	Spontaneous heme iron utilization mutant of *P. shigelloides* 9	This study
V. cholerae		
CA401	Wild type; classical strain	15
CA40130	Vibriobactin synthesis mutant of CA401	17
DHH-11	TonB⁻ mutant of CA40130	20
E. coli		
1017	*entf*::Tn*5* mutant of HB101; F⁻ Δ*(gpt-proA)62 leuB6 glnV44 ara-14 galK2 lacY1* Δ*(mcrC-mrr) rpsL20* Str^r *xyl-5 mtl-1 recA13*	S. M. Payne
RK1065L	*hemA*	R. Kadner
DHE-1	RK1065L *entB*	This study
DH5α	F⁻ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ*(argF-lacZYA)U169* (φ80Δ*lacZ* M15) λ⁻	18
KP1032	*tonB*::Kan mutant of W3110 F⁻ IN*(rrnD-rrnE)1*	25
Plasmids		
pHPS1	Amp^r; 30-kb (partial) *Sau*3A fragment of *P. shigelloides* 9 DNA cloned into pJB8; carries *hugAWXZ, tonB, exbBD*	9
pHPS6	Amp^r; 48-kb (partial) *Sau*3A fragment of *P. shigelloides* 9 DNA cloned into pJB8; *hugBCD*	P. Daskaleros
pHUG1	Cm^r; pHUG2 without the 1.2-kb *Eco*RV-*Hin*dIII fragment; contains *P. shigelloides tonB*	This study
pHUG2	Cm^r; contains 2.7-kb *Ase*I-*Hin*dIII fragment of pHUG10 cloned into the *Eco*RV-*Hin*dIII site of pACYC184; contains *P. shigelloides tonB* and *exbBD*	This study
pHUG2.1	Cm^r, Kn^r; pHUG2 with 1.2 kb-*Pst*I fragment containing kanamycin cassette from pUC4K cloned into *Pst*I site of pHUG2; disrupts *P. shigelloides tonB*	This study
pHUG3	Tet^r; 2.5-kb *Pst*I fragment from pHUG10 cloned into *Pst*I site of pAT153; contains *hugA*	This study
pHUG3.1	Cb^r; 2.5-kb *Pst*I fragment from pHUG3 cloned into *Pst*I site of pWSK29	This study
pHUG3.2	Tet^r; 2.5-kb *Pst*I fragment from pHUG3 cloned into *Pst*I site of pLAFR3	This study
pHUG4	Amp^r; 7.5-kb *Hin*dIII-*Psh*AI fragment of pHUG10 cloned into *Hin*dIII-*Sma*I site of pWSK29; contains *hugA, hugWXZ, tonB, exbBD*	This study
pHUG7	Amp^r; 7-kb *Hin*dIII-*Nhe*I fragment from pHUG10 cloned into *Hin*dIII-*Xba*I site of pWSK29; contains *hugA* and *hugWXZ*	This study
pHUG8	Cm^r; pHUG10 without the 2.4-kb *Bsi*WI fragment; contains *hugA, tonbB, exbBD*	This study
pHUG10	Cm^r; 10-kb *Hin*dIII fragment of pHPS1 cloned into pACYC184; contains *hugA, hugWXZ, tonB, exbBD*	This study
pHUG10.1	Tet^r; 10-kB *Hin*dIII fragment of pHUG10 cloned into pLAFR3	This study
pHUG16	Tet^r; 6-kB *Eco*RI fragment of pHPS6 cloned into pACYC184; contains *exbBD, hugBCD*	This study
pHUG*lac*1	Amp^r; 2.3-kb *Bam*HI fragment from pHUG10 cloned into pQF50; contains putative *hugAW* promoter in front of *lacZ*	This study
pTON*lac*1	Amp^r; 2.3-kb *Bam*HI fragment from pHUG10 cloned into pQF50 in opposite orientation as in pHUG*lac*1; contains putative *tonB* promoter in front of *lacZ*	This study
pTEE1	Cm^r; 2.1-kb PCR fragment containing *V. cholerae, tonB1, exbB1D1* cloned into pACYC184	38
pCOS3	Tet^r; 30-kb partial *Sau*3A1 fragment of *V. cholerae* DNA cloned into pLAFR3; contains *tonB2, exbB2D2*	38
pHUT3	Amp^r; 3-kb *Sal*I-*Hin*dIII fragment of *V. cholerae* DNA cloned into pAT153; contains *hutA*	20
pHUT10	Cm^r; 10.3-kb *Hin*dIII fragment of *V. cholerae* DNA cloned into pACYC184; contains *tonB1, exbB1D1, hutBCD* and *hutWXZ*	20
pAC1	Cm^r; pACYC184 without the *Bam*HI-*Sal*I fragment containing the tetracycline resistance gene; used to provide antibiotic resistance to DPH-2 for triparental matings	This study
pETONBX	Amp^r; PCR fragment containing *E. coli tonB* cloned into pWKS30	A. Mey
pACYC184	Cm^r, Tet^r; plasmid vector	6
pAT153	Amp^r, Tet^r; plasmid vector	56
pWSK29	Amp^r; low-copy-number plasmid vector	59
pQF50	Amp^r; plasmid vector for construction of promoter *lac* fusions	12
pUC4K	Kan^r, Amp^r; vector containing kanamycin cassette	Pharmacia
pLAFR3	Tet^r; mobilizable cosmid cloning vector	49

β-Galactosidase assays. β-Galactosidase assays were performed on mid-log-phase cultures as described by Miller (36) on *E. coli* DH5α that had been transformed with pHUG*lac*1 or pTON*lac*1. Overnight L broth cultures were washed twice in M9 medium (36) and diluted 1:25 into M9 medium containing 0.3% Casamino Acids deferrated as described by Pugsley and Reeves (43). Low-iron medium contained 300 μg of EDDA per ml, and high-iron medium contained 40 μM FeSO$_4$. Independent experiments were conducted on each strain grown under the same conditions on three separate occasions.

Growth assays. To detect the utilization of heme as an iron source, overnight cultures of *E. coli* 1017 containing the indicated plasmid(s) were diluted 1 to 1,000 into L broth or L broth containing 200 μg of EDDA per ml with or without hemin. Absorbance at 600 nm was measured after 15 to 18 h of growth. An

absorbance of 1.4 or above was considered a positive result and 0.5 or below a negative result. In bioassays to detect utilization of heme as a porphyrin source, mid-log-phase cultures of DHE-1 were washed with saline and seeded into L agar with kanamycin at 5×10^4 cells/ml. Five microliters of 20-mg/ml ALA or 95 μM heme was spotted onto the media, and zones of growth were measured after 15 h. Bioassays to detect utilization of heme as an iron source in DHE-1 were performed in the same manner, except the cells were seeded into L agar containing ALA and EDDA (300 μg/ml). Other heme iron utilization bioassays were performed in manners similar to those of the three assays described above, with specific details being provided in the appropriate table.

Electroporation and triparental matings. The electroporation of *P. shigelloides* was performed as previously described (38). Triparental mating with the mobilizing strain MM294/pRK2013 also was used to transfer recombinant plasmids into *P. shigelloides*.

DNA sequencing and analysis. The DNA sequence of both strands of the insert in pHUG10 and other recombinant plasmids was determined with an ABI Prism 377 DNA sequencer from Applied Biosystems and was analyzed with the DNA Strider program (34). The BLAST program of the National Center for Biotechnology Information (1) was used to determine homologies of the deduced amino acid sequences, and MacVector ClustalW was used to determine protein identity and similarity.

Nucleotide sequence accession number. The nucleotide and amino acid sequences corresponding to this region can be found under GenBank/EMBL accession no. AY008342.

RESULTS

Nucleotide sequence analysis of *P. shigelloides* heme iron utilization genes. The genetic organization of the *P. shigelloides* heme iron utilization locus was determined by DNA sequence analysis of pHUG10, a plasmid which enables *E. coli* 1017 to utilize heme as an iron source, and of pHUG16, which contains *hugBCD*. Our analyses indicated the presence of 10 open reading frames (ORFs) which spanned approximately 10,000 nucleotides. Figure 1A shows the genetic organization of the region. The calculated molecular weights and pIs for these proteins and the percent identity and similarity of the predicted proteins to selected proteins in other organisms are shown in Table 2.

The *P. shigelloides* *hugA* gene encodes a protein with a predicted leader sequence of 41 amino acids. HugA has homology with a number of outer membrane heme receptors (Table 2). In addition, HugA contains sequences found by Bracken et al. (3) to be present in most heme receptors but absent in outer membrane receptors not involved in heme uptake. Figure 1B shows a comparison of some of these sequences in *Yersinia enterocolitica* HemR and *P. shigelloides* HugA. Two histidines (residues 128 and 461 in HemR) (Fig. 1B) required for HemR receptor function (3) are conserved in HugA. HugA also contains the FRAP and NPNL boxes and two conserved glutamic acids on the carboxy-terminal side of the NPNL box (Fig. 1B). A putative TonB box with the sequence NEVLVTA is located 17 amino acids from the predicted amino terminus of the mature HugA. This sequence is similar to those of the putative TonB boxes in the *V. cholerae* heme receptor, HutA (DEVVVST) (21), and in the *V. vulnificus* heme receptor, HupA (DEVVVSA) (30). Among the three TonB boxes, there is identity in four of seven amino acids.

Downstream of *hugA* are three genes (*hugWXZ*) which are transcribed in the same direction as *hugA* (Fig. 1A). The first of these genes, *hugW*, which overlaps the stop codon of *hugA*, encodes a protein which shares homology to ORFs in other heme transport loci (Table 2) and has weaker homology with the *Salmonella enterica* serovar Typhimurium protein HemN,

FIG. 1. (A) Genetic and restriction enzyme map of cloned DNA from *P. shigelloides* heme iron utilization locus and identification of sequences required for utilization of heme as an iron source or as a porphyrin source. Genes carried by pHUG10 and pHUG16 are shown with horizontal arrows indicating the direction of transcription. Putative promoters are indicated with vertical bent arrows. Downstream of *hugZ* and *hugD* are regions of dyad symmetry indicated with two vertical lines with a circle at the top. The restriction enzyme sites shown are as follows: H, *Hin*dIII; Ps, *Psh*A1; Bw, *Bsi*WI; P, *Pst*I; A, *Ase*I; N, *Nhe*I; R, *Eco*RI; RV, *Eco*RV. Not all of the *Pst*I, *Eco*RI, *Eco*RV, and *Psh*AI sites are shown. The heme iron and heme porphyrin assays are described in Materials and Methods. ND, not determined. (B) Amino acid comparison of highly conserved heme receptor sequences in *Y. enterocolitica* HemR and *P. shigelloides* HugA. The numbers to the left of the sequences indicate the positions of the first amino acids. An asterisk below the sequence indicates identical amino acid residues and a period indicates conservative substitutions. The bold-faced sequences are those found by Bracken et al. (3) to be present in most heme receptors. Histidines 128 and 461 in HemR are important for the proper function of the protein (3).

an oxygen-independent form of coproporphyrinogen oxidase. Coproporphyrinogen oxidases convert coproporphyrinogen III into protoporphyrin IX, a late step in heme biosynthesis.

The gene for HugX is 224 nucleotides downstream of *hugW*. HugX has homology to ORFs linked to heme transport systems in other organisms, including the *V. cholerae* HutX and the *S. dysenteriae* ShuX (Table 2), but no homology with ORFs of known function.

HugZ is 165 nucleotides downstream of *hugX*. HugZ shares homology to HutZ, an ORF linked to the *V. cholerae* heme utilization locus, and to *Haemophilus influenzae* hypothetical protein HI0854 (Table 2), neither of which has a known function. A potential stem-loop structure is located 143 nucleotides downstream of *hugZ* and may serve as a transcriptional terminator.

The other six ORFs are transcribed in the opposite direction of *hugAWXZ* (Fig. 1A). *P. shigelloides* TonB shares homology to TonB proteins in other organisms (Table 2). The *exbB* gene is 67 nucleotides downstream of *tonB*, and *exbD* overlaps *exbB* by 155 nucleotides. *P. shigelloides* ExbBD proteins share homology with ExbBD proteins in several organisms (Table 2). The start codon for the *P. shigelloides* hugB overlaps the stop codon for *exbD*. HugB is similar in amino acid sequence to

TABLE 2. Proteins with homology to products of *P. shigelloides* heme utilization locus

P. shigelloides protein (MW)[a] (pI)[b]	Homologue (reference)	Amino acid identity (%)	Amino acid similarity (%)
HugA (71,900) (5.23) (mature)	*H. influenzae* HxuC (8)	36	50
	Y. enterocolitica HemR (50)	28	42
	S. dysenteriae ShuA (37)	28	41
	E. coli O157:H7 ChuA (54)	28	41
	Yersinia pestis HmuR (53)	28	41
	V. cholerae HutA (21)	25	41
HugW (50,200) (6.50)	*V. cholerae* HutW (19)[c]	51	68
	V. parahaemolyticus PhuW (40)	51	66
	S. enterica serovar Typhimurium HemN (62)	21	36
HugX (17,500) (7.36)	*Y. pestis* hypothetical protein X[d]	38	54
	V. cholerae HutX (19)[e]	37	53
	S. dysenteriae ShuX (60)	34	50
HugZ (21,000) (5.11)	*V. cholerae* HutZ (19)[f]	60	76
	H. influenzae hypothetical protein HI0854 (14)	21	39
TonB (30,200) (10.18)	*V. cholerae* TonB1 (38)	32	48
	V. parahaemolyticus TonB (40)	31	42
	P. aeruginosa TonB2 (63)	26	39
	E. coli TonB (42)	24	34
	V. cholerae TonB2 (38)	20	29
ExbB (29,700) (10.64)	*V. cholerae* ExbB1 (38)	41	56
	V. parahaemolyticus ExbB (40)	40	54
	V. cholerae ExbB2 (38)	18	30
ExbD (15,700) (4.52)	*V. cholerae* ExbD1 (38)	41	63
	Pseudomonas putida TolR[g]	22	46
	V. cholerae ExbD2 (38)	21	31
HugB (32,800) (8.99)	*V. cholerae* HutB (38)	34	51
	Y. enterocolitica HemT (51)	30	50
	Y. pestis HmuT (53)	30	49
HugC (36,800) (10.71)	*V. cholerae* HutC (38)	52	70
	P. aeruginosa PhuU[h]	50	66
	Y. enterocolitica HemU (51)	45	65
HugD (28,800) (8.45)	*V. cholerae* HutD (38)	48	68
	Y. pestis HmuV (53)	41	60
	Y. enterocolitica HemV (51)	39	60

[a] MW, calculated molecular weight.
[b] Calculated pI.
[c] ORF VCA0909 (19).
[d] GenBank accession number T12066.
[e] ORF VCA0908 (19).
[f] ORF VCA0907 (19).
[g] GenBank accession number X74218.
[h] GenBank accession number AAC13286.

periplasmic binding proteins found in several bacteria (Table 2). The *P. shigelloides* *hugC* begins 13 nucleotides downstream from *hugB*, and *hugD* begins 11 nucleotides downstream from *hugC*. HugCD share homology with a number of inner membrane permeases in other organisms (Table 2), and HugD contains Walker motif A, GPNGTGKS (amino acids 31 to 38), and motif B, LLMLDEPT (amino acids 168 to 175) (57), suggesting that it is the ATPase component of the complex. A potential stem-loop structure is located 56 nucleotides downstream of *hugD*.

Predicted promoters for heme utilization genes. Each of the genes in the *P. shigelloides* heme utilization locus except *hugW*,

exbD, and *hugBCD* appears to contain its own promoter (Fig. 1A). The predicted promoter for *hugZ* contains a potential Fur box upstream of the putative −35 region (Fig. 2A). The putative promoters for *hugAW* and *tonB* are divergent overlapping promoters and also contain a sequence that resembles a Fur box (Fig. 2B). The putative Fur box, which overlaps the −10 region of each promoter, shares 12 of 19 nucleotides with the consensus sequence of the *E. coli* Fur box (10) and 13 of 19 nucleotides with the *V. cholerae viuA* downstream Fur box (5) (data not shown). To assess regulation of the predicted *hugAW-tonB* promoters, the *hugA-tonB* intergenic region was cloned in both orientations upstream of the promoterless *lacZ* gene in

A

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        ←
952  TATTAGTCTAATTTACTGCCCTGATAAGGCTATTCACGTTGTCGATTTTTATTATC
     ATAATCAGATTAAATGACGGGACTATTCCGATAAGTGCAACAGCTAAAAATAATAG
            hugZ                    hugZ
          -10 region             -35 region    Fur box
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B

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      tonB                    tonB
    -35 region             -10 region              →
5309  TTGAATGCGAATGCAAATAATTTTTCATTTGATCTCAGGTGTCAT
      AACTTACGCTTACGTTTATTAAAAGTAAACTAGAGTCCACAGTA
                  Fur box                          hugA
                                                 -35 region
              ↙                 hugA
                              - 10 region
```
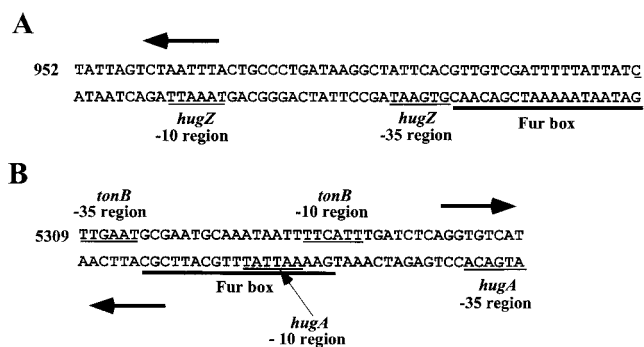
FIG. 2. Predicted promoters for *hugZ*, *hugAW*, and *tonB*. The DNA sequences of the putative *hugZ* promoter (A) and of the putative *hugAW-tonB* promoters (B) are shown. The numbers to the left of the sequences indicate the positions of the first nucleotide in the sequences. The heavy horizontal arrows indicate the direction of transcription. The predicted −10 and −35 regions are underlined and labeled. The putative Fur boxes are indicated with a heavy black line underneath the sequence.

pQF50 to create pHUG*lac*1 and pTON*lac*1. β-Galactosidase assays were performed on *E. coli* DH5α containing the indicated plasmids following growth in high- or low-iron medium. When either transformed strain was grown in high-iron medium, the level of β-galactosidase activity was approximately 30 U. When the strains were grown under low-iron conditions, activity increased approximately 19-fold for both strains (577 in DH5α/pHUG*lac*1 and 558 in DH5α/pTON*lac*1). These data indicate that promoters in the *hugA-tonB* intergenic region are iron regulated.

Reconstitution of the *P. shigelloides* heme iron utilization system in *E. coli* 1017. To determine which genes are necessary for heme iron utilization, the Ent⁻ *E. coli* strain 1017 containing subclones of pHUG10 was tested for growth in L broth containing heme as the sole iron source (Fig. 1A). *E. coli* 1017/pHUG10 grew well in this medium, indicating that *hugBCD* are not required for heme iron utilization. *E. coli* 1017/pHUG4 also grew in media with heme as the iron source, indicating that sequences downstream of *hugZ* are not required for heme iron utilization. *E. coli* 1017/pHUG3, containing only *hugA*, or 1017/pHUG7, containing *hugAWXZ*, did not utilize heme as an iron source, indicating that the *P. shigelloides* TonB system genes are required. This was confirmed when 1017 containing both pHUG7 and pHUG2 was able to utilize heme. Heme iron utilization did not occur when 1017/pHUG7 was transformed with pHUG2.1, which contains a kanamycin cassette that disrupts *tonB*, or when the *exbBD* genes from pHUG2 were removed (pHUG1). These data indicate that *P. shigelloides* TonB requires ExbBD to function and that *E. coli* ExbBD cannot substitute for the *P. shigelloides* counterparts. To determine if the genes downstream of *hugA* are required for heme iron utilization, *E. coli* 1017 transformed with pHUG8, which contains *hugA, tonB,* and *exbBD* but is missing *hugWXZ*, was tested, and the strain failed to utilize heme.

Genes required for utilization of heme as a porphyrin source. We wanted to determine if the genes needed for utilization of heme as an iron source are also required for utilization of heme that is incorporated in intact form into heme proteins. Bioassays were conducted with DHE-1, a *hemA entB*

mutant of *E. coli*, which cannot synthesize heme in the absence of the precursor ALA and cannot transport heme to meet the porphyrin requirement. In the assay, the strains were seeded into L agar, and ALA or heme was spotted onto the plates.

DHE-1/pHUG10 grew around the heme spot, indicating that pHUG10 contained the necessary genes for heme to be utilized as a porphyrin source (Fig. 1A). DHE-1 containing pHUG8, which has only *hugA, tonB,* and *exbBD*, also could utilize heme as a porphyrin source, suggesting that *hugWXZ* are not required for utilization of heme as a porphyrin source. The strain transformed with pHUG3.1, which carries *hugA*, could not utilize heme as a porphyrin source. These data indicate that the heme receptor gene and the TonB system genes are required for the utilization of heme as a porphyrin source.

***hugW, -X,* or *-Z* is needed to prevent heme toxicity.** We also seeded the strains into iron-restricted medium containing ALA and supplied heme as an iron source. As expected, *E. coli* DHE-1/pHUG10 grew well in the assay, exhibiting a zone of growth of approximately 12 mm. In contrast, DHE-1/pHUG8 grew poorly and appeared to be inhibited by the higher concentration of heme present in the center of the spot. The strain exhibited a 14-mm zone of inhibition surrounded by a very faint 3-mm zone of growth. This suggested that the high level of heme was toxic to the cells.

We also determined the effect of several different concentrations of heme on the growth of DHE-1/pHUG8 and found that at higher heme concentrations, the zone of inhibition increased in diameter (data not shown). Because it is possible that growing the strain in media containing both ALA and heme resulted in the accumulation of toxic intermediates in the heme biosynthetic pathway that would not be present when either heme or ALA were present alone, the heme iron assay was performed with no ALA in the L agar. Growth around the heme spots was essentially the same as that observed when ALA was supplied in the L agar (data not shown). Growth inhibition in the presence of heme was also observed in a HemA⁺ strain (*E. coli* 1017), indicating that the inhibition is unlikely to be associated with the *hemA* mutation. These data suggest that *hugW, -X,* or *-Z* is necessary to prevent heme toxicity when heme is supplied as an iron source. It is not clear why heme supplied as an iron source, but not as a porphyrin source, is toxic to the cells. This could be due to the fact that when heme was supplied as an iron source, the cells are grown under low-iron conditions, which increases the expression of heme utilization proteins and which may allow higher levels of heme to enter the cytoplasm.

Determination that HugA is a heme receptor. Our sequencing data indicated that HugA shares homology with a number of heme receptors, including *V. cholerae* HutA. Thus, we determined if HugA could function as a heme receptor by testing its ability to substitute for *V. cholerae* HutA. The *V. cholerae* heme utilization system can be reconstituted in *E. coli* 1017 by transforming the strain with recombinant plasmids pHUT3, which carries *hutA*, and pHUT10, which carries *tonB1, exbB1D1, hutBCD,* and *hutWXZ*. When *E. coli* 1017/pHUT3/pHUT10 was tested for growth in L broth with EDDA and heme, the strain utilized heme as an iron source, whereas *E. coli* 1017/pHUG10 could not, confirming previously published results (20) (Table 3). To determine if HugA can substitute for HutA, we moved pHUG3, which carries the *P. shigelloides*

TABLE 3. *P. shigelloides* HugA functions as a heme receptor in *E. coli*

E. coli 1017 transformed with plasmid:	Absorbance of cultures grown in[a]:		
	L broth	L broth with EDDA	L broth with EDDA plus heme
pHUT10/pHUT3 (*V. cholerae* system)	1.20	0.05	0.95
pHUT10/pHUG3 (hybrid system with *P. shigelloides hugA*)	1.20	0.06	1.25
pHUT10 (no receptor gene)	1.02	0.05	0.08

[a] Absorbance at 650 nm was taken after 18 h of growth.

TABLE 5. Complementation of an *E. coli tonB* mutant with the *P. shigelloides* TonB system genes

E. coli KP1032 transformed with plasmid:	Absorbance of cultures grown in[a]:	
	L broth	L broth with EDDA (enterobactin utilization)
No plasmid (TonB⁻)	1.49	0.23
pETONBX (*E. coli tonB*)	1.48	1.51
pHUG2 (*P. shigelloides* TonB system)	1.55	1.34
pHUG1 (*P. shigelloides tonB*)	1.50	0.03

[a] Absorbance was taken at 600 nm after 17 h of growth.

gene *hugA*, into 1017/pHUT10 to create a chimeric heme iron utilization system. This strain grew as well as 1017/pHUT3/pHUT10 in L broth with EDDA and heme, indicating that *hugA* encodes a protein that can replace the function of the *V. cholerae* HutA.

To confirm these results, a heme iron utilization mutant of *P. shigelloides* 9 was isolated as described in Materials and Methods. The mutant DPH-2 failed to grow with heme or hemoglobin as an iron source (Table 4). DPH-2 was complemented with pHUG10.1, which contains all the *P. shigelloides* heme iron utilization genes characterized in this paper except *hugBCD*, or with pHUG3.2, which contains only *hugA*. The mutant also was complemented with pHUT2, which contains *V. cholerae hutA*. However, pHUT4, which contains *V. cholerae tonB1, exbB1D1, hutBCD*, and *hutWXZ* but not *hutA* could not utilize heme (Table 4). These data further support our contention that *hugA* encodes the *P. shigelloides* heme receptor. The results also indicate that *hugA* can be added to the list of *P. shigelloides* genes required for heme iron utilization.

Complementation of *tonB* mutants with *P. shigelloides* TonB system genes. Because *P. shigelloides* TonB and ExbBD resemble proteins found in TonB systems from other organisms, we wanted to determine if these proteins function as a TonB system. We moved the *P. shigelloides* TonB system on pHUG2 or *E. coli tonB* on pETONBX into KP1032, an *E. coli tonB*

TABLE 4. Characterization of a *P. shigelloides* heme utilization mutant

P. shigelloides strain	Growth with the iron source[a]:		
	Heme	Hemoglobin	FeSO₄
P. shigelloides 9 (wild type)	+[b]	+	+
DPH-2 (heme utilization mutant)	−[c]	−	+
DPH-2/pHUG10.1 (contains *hugAWXZ, tonB, exbBD*)	+	+	+
DPH-2/pHUG3.2 (contains *hugA*)	+	+	+
DPH-2/pHUT2 (contains *V. cholerae hutA*)	+	+	+
DPH-2/pHUT4 (contains *V. cholerae tonB1, exbB1D1, hutBCD, hutWXZ*)	−	−	+

[a] Strains were seeded into L agar containing 50 μg of EDDA per ml at 10⁵ cells/ml. Five microliters of 40 μM heme, 10 μM hemoglobin, or 10 mM FeSO₄ was spotted onto the plates, and the plates were incubated for 15 h.
[b] A plus represents a zone of growth of at least 8 mm around the spot of FeSO₄ and 10 mm around the spots of heme and hemoglobin.
[c] A minus indicates no detectable growth.

mutant, and performed complementation assays on the strains. The strains were grown in low-iron medium, which tests for the ability to acquire iron via the siderophore enterobactin, a TonB-dependent process. *E. coli* KP1032/pHUG2 grew as well in low-iron medium as the positive control, KP1032/pETONBX (Table 5), indicating that the *P. shigelloides* TonB system complements an *E. coli tonB* mutant. However, when the *P. shigelloides tonB* gene without *exbBD* was provided on pHUG1, the strain failed to grow. The fact that the *P. shigelloides* TonB by itself could not restore function but the entire TonB system could suggests that *P. shigelloides* TonB cannot function with the *E. coli* ExbBD proteins but that it can function with the enterobactin receptor in *E. coli*.

We also tested pHUG2 for complementation of DHH-11, a TonB⁻ strain of *V. cholerae*. DHH-11/pHUG2 could acquire iron from heme and the siderophore vibriobactin, which are TonB-dependent processes, whereas the untransformed strain could not (data not shown).

Salt sensitivity of *P. shigelloides* TonB. Seliger et al. (46) reported that high levels of salt diminish the function of *V. cholerae* TonB2 but have no impact on TonB1 function. This is thought to occur because TonB1 is longer than TonB2 (244 amino acids versus 206 amino acids). Thus, TonB1 can extend across the periplasm and interact with the receptor under high-salt conditions when the periplasm may become larger due to possible shrinkage of the cytoplasm. To determine if *P. shigelloides* TonB, which is 288 amino acids in length, can also function under high-salt conditions, we moved plasmids containing various TonB systems into *E. coli* 1017/pHUG7 (contains *hugAWXZ*). The following TonB systems were moved into the strain: the *P. shigelloides* TonB system on pHUG2, the *V. cholerae* TonB1 system on pTEE1, and the *V. cholerae* TonB2 system on pCos3. All the strains grew well in high-salt L broth, which showed that high levels of salt in iron-rich media did not inhibit growth (Table 6). The strains also grew in low-salt medium with heme as the iron source, which indicated that each of the three TonB proteins could interact with *P. shigelloides* HugA to allow heme to enter the cell (Table 6). When the strains were tested for their ability to utilize heme as an iron source in high-salt media, substantial growth was observed with *E. coli* 1017/pHUG7 containing the *P. shigelloides* TonB system or the *V. cholerae* TonB1 system but not in the strain containing the *V. cholerae* TonB2 system. These data

TABLE 6. Ability of *P. shigelloides* TonB and *V. cholerae* TonB1 and TonB2 to function in high-salt medium

E. coli 1017 transformed with plasmid:	Growth in media containing[a]:				
	L broth		L broth with EDDA (low salt)	L broth with EDDA plus heme	
	Low salt	High salt		Low salt	High salt
pHUG7/pHUG2 (*P. shigelloides* TonB)	1.94	1.56	0.02	1.77	1.37
pHUG7/pTEE1 (*V. cholerae* TonB1)	2.20	1.77	0.00	1.90	1.40
pHUG7/pCos3 (*V. cholerae* TonB2)	2.03	1.76	0.05	1.30	0.40

[a] Strains were grown in L broth containing 170 mM NaCl (low salt) or 625 mM NaCl (high salt) containing no EDDA or containing 200 μg of EDDA per ml. with or without hemin. Absorbance at 600 nm was taken after 18 h of growth.

suggest that *P. shigelloides* TonB is like *V. cholerae* TonB1 in its ability to function under high-salt conditions.

DISCUSSION

Our analyses of the *P. shigelloides* heme iron utilization system indicates it contains an outer membrane receptor, HugA, which shares significant homology to other heme iron receptors. HugA also contains several motifs that are present in most heme receptors but are absent in receptors for other ligands. Our data indicate that HugA functions as a heme receptor in that it is interchangeable with the *V. cholerae* heme receptor in both a *hugA* mutant of *P. shigelloides* and in an *E. coli* strain that contains a hybrid heme iron utilization system consisting of *hugA* and *V. cholerae tonB1, exbB1D1, hutBCD*, and *hutWXZ*. The ability of these two heme receptors to function interchangeably may be due to the similar amino acid sequences of their TonB boxes (NEVLVTA in HugA and DEVVVST in HutA). The TonB box is the region of an outer membrane receptor that is thought to physically interact with the TonB protein. Other regions that the two proteins share in common also may play a role in allowing HugA and HutA to be interchangeable, as the two proteins share significant homology overall (Table 2).

P. shigelloides also has a TonB system which is required for heme iron utilization. The TonB system genes are arranged in the same order as those in the *V. cholerae* heme iron utilization system (*tonB, exbB, exbD*) and are linked to other genes specifically required for heme iron utilization. Linkage of a ligand transport system to *tonB* is unusual. *P. shigelloides, V. cholerae* (38), and possibly *Vibrio parahaemolyticus* (40) are the only organisms characterized to date which share this feature. The fact that these three organisms have TonB system genes linked to other heme iron utilization genes suggests that the genes were acquired simultaneously by horizontal gene transfer, and that at least initially these TonB systems were specific for heme iron utilization.

The *P. shigelloides* TonB and ExbBD sequences most closely resemble their counterparts in the *V. cholerae* TonB1 system. *P. shigelloides* TonB also is similar in size to *V. cholerae* TonB1 and like TonB1 can function in high-salt conditions. However,

the *P. shigelloides* TonB system differs from the *V. cholerae* TonB1 system in that it can complement an *E. coli* TonB mutant, whereas the *V. cholerae* TonB1 system cannot (38). *V. cholerae* has a second TonB system (38) which, like the *P. shigelloides* system, complements an *E. coli tonB* mutant. Thus, it appears that the *P. shigelloides* TonB system shares features with both TonB systems in *V. cholerae*.

O'Malley et al. (40) suggested that *V. parahaemolyticus* and *Vibrio alginolyticus* have two *tonB* genes similar to those identified in *V. cholerae*, and two *tonB* genes have been identified in *Pseudomonas aeruginosa* (63). It is not clear if *P. shigelloides* also has two different *tonB* genes. No signal was detected in Southern blots of *P. shigelloides* chromosomal DNA probed with *V. cholerae tonB2*. This suggests that *P. shigelloides* does not have a *tonB2* homologue but does not rule out the possibility that a second *tonB* is present that cannot be detected with the *V. cholerae tonB2* probe at low stringency.

Many heme iron utilization systems characterized to date have genes encoding periplasmic and inner membrane permeases, which move the heme across the periplasm and into the cytoplasm. In *V. cholerae* these genes (*hutBCD*) are located downstream of *exbD1*. *P. shigelloides* also has homologues to these genes (*hugBCD*) which are located downstream of *exbD*. However, *hugBCD* are not needed to reconstitute the *P. shigelloides* heme iron utilization system in *E. coli* 1017, whereas *V. cholerae hutBCD* are needed to reconstitute the *V. cholerae* system in the same *E. coli* strain (38).

The role of the proteins encoded by *P. shigelloides hugWXZ* is not clear. Several bacterial heme iron utilization systems contain homologues of one or more of these proteins (Table 2), but functions have yet to be assigned to these proteins. Our results indicate that these three genes are not required for utilization of heme as a porphyrin source but are needed when heme is used as an iron source. When the *P. shigelloides* heme utilization system is reconstituted in *E. coli* in the absence of *hugWXZ*, heme supplied as an iron source is toxic to the cells. Stojiljkovic and Hantke (51) observed a similar result when they reconstituted the *Y. enterocolitica* heme iron utilization system in *E. coli*. In those experiments, *hemS* was required for utilization of heme as an iron source but not for utilization of heme as a porphyrin source, and heme toxicity was observed in the absence of *hemS*. The researchers hypothesized, but have not confirmed, that HemS breaks down the heme and releases iron into the cytoplasm. One or more of the proteins encoded by *hugWXZ* could serve a similar function in *P. shigelloides*, although none were found to share homology with HemS. It is also possible that these proteins may not be involved in breaking down the heme but rather in controlling the level of heme that enters the cytoplasm of the cell. The proteins could also be involved in heme storage, and their absence could result in accumulation of toxic levels of heme in the cytoplasm.

While *P. shigelloides* and *V. cholerae* have heme iron utilization systems that are similar to one another, the two organisms appear to be very different in the number of strategies each possesses for acquiring iron in the host. *V. cholerae* has several methods of acquiring iron from the host. *V. cholerae* utilizes the siderophores vibriobactin (17), ferrichrome (17), schizokinen (46), and enterobactin (61). *P. shigelloides* may not exhibit the same versatility. Daskaleros et al. (9) demonstrated that *P. shigelloides* does not synthesize a siderophore and that it can-

not utilize vibriobactin or enterobactin. We conducted additional tests and found that it could not utilize ferrichrome or schizokinen or be crossfed by *V. parahaemolyticus*, which produces vibrioferrin, or by *V. alginolyticus*, which produces a yet-to-be-characterized siderophore (data not shown). Thus, the organism can use heme but not siderophores as an iron source. One could speculate that the absence of siderophore uptake systems in *P. shigelloides* may have a negative impact on its ability to survive in the environment and to infect potential hosts. This may explain why *P. shigelloides* appears to be less successful than *V. cholerae* in causing disease in a large number of humans.

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