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Characterization of Heme Uptake Cluster Genes in the Fish Pathogen *Vibrio anguillarum*

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Vibrio anguillarum **can utilize hemin and hemoglobin as sole iron sources. In previous work we identified HuvA, the *V. anguillarum* outer membrane heme receptor by complementation of a heme utilization mutant with a cosmid clone (pML1) isolated from a genomic library of *V. anguillarum*. In the present study, we describe a gene cluster contained in cosmid pML1, coding for nine potential heme uptake and utilization proteins: HuvA, the heme receptor; HuvZ and HuvX; TonB, ExbB, and ExbD; HuvB, the putative periplasmic binding protein; HuvC, the putative inner membrane permease; and HuvD, the putative ABC transporter ATPase. A *V. anguillarum* strain with an in-frame chromosomal deletion of the nine-gene cluster was impaired for growth with heme or hemoglobin as the sole iron source. Single-gene in-frame deletions were constructed, demonstrating that each of the *huvAZBCD* genes are essential for utilization of heme as an iron source in *V. anguillarum*, whereas *huvX* is not. When expressed in *Escherichia coli hemA* (strain EB53), a plasmid carrying the gene for the heme receptor, HuvA, was sufficient to allow the use of heme as the porphyrin source. For utilization of heme as an iron source in *E. coli ent* (strain 101ESD), the *tonB exbBD* and *huvBCD* genes were required in addition to *huvA*. The *V. anguillarum* heme uptake cluster shows some differences in gene arrangement when compared to homologous clusters described for other *Vibrio* species.**

Iron is an essential element for most bacteria, serving as a cofactor in key metabolic processes such as nucleotide biosynthesis, electron transfer, and energy transduction. Most bacterial pathogens require iron for growth and to establish an infection, and thus they have developed efficient mechanisms to obtain iron from the host (32). The small amounts of extracellular iron are quickly bound by high-affinity carrier proteins such as transferrin in serum and lactoferrin in secretions. *Vibrio anguillarum* is the etiological agent of a septicemic disease known as vibriosis, which affects a large number of marine fish species. Within the 10 O serogroups described by Sørensen and Larsen (35), only serotypes O1 and O2 and, to a lesser extent, serotype O3 are considered important fish pathogens. Although *V. anguillarum* is the best known fish pathogen of the genus *Vibrio*, the nature of its virulence mechanisms is not thoroughly understood. Strains of pathogenic *V. anguillarum* serotypes can acquire iron by the production and secretion of siderophores (2, 4, 16, 19, 38). Heme and some heme-containing proteins, including hemoglobin and hemoglobin-haptoglobin, can also be used as iron sources by a siderophore-independent mechanism (23, 24).

In this way, many gram-negative pathogens have the ability to obtain iron through utilization of free heme or heme proteins from the host tissues (7, 18), and heme utilization genes have been identified in numerous species, including *Yersinia enterocolitica* (36, 37), *Vibrio cholerae* (13, 26, 29), *Escherichia coli* O157 (39), *Vibrio vulnificus* (20), *Plesiomonas shigelloides* (14), and *Shigella dysenteriae* (28) among others. Specific receptors are involved in heme binding and transport. Receptor-mediated uptake of heme includes translocation of the ligands into the periplasm by an energy-dependent process that requires a functional TonB system (30, 36). The TonB protein, which is anchored in the cytoplasmic membrane and associated with two accessory proteins, ExbB and ExbD, spans the periplasm and interacts with the ligand-loaded receptor. The TonB-ExbBD system is believed to be involved in transducing the energy of the proton motive force of the cytoplasmic membrane into transport energy required by the receptor. The pernicious oxidative effects of free heme dictate the presence of a periplasmic binding protein to transport heme across the periplasmic compartment. Transport of heme or iron across the cytoplasmic membrane is driven by ATP hydrolysis, and an ATP-binding cassette (ABC) transporter is commonly involved in transport through the cytoplasmic membrane (17).

In previous studies we identified HuvA, the outer membrane receptor involved in heme uptake in *V. anguillarum*. The *huvA* gene was isolated from a *V. anguillarum* H-775-3 cosmid library by its ability to restore heme utilization in 101ESD, an *E. coli* mutant strain that fails to grow under iron-limiting conditions and cannot use heme as an iron source (25). A TonB-ExbB-ExbD system has also been recently found in *V. anguillarum*. It was observed that a *tonB* mutant strain was still able to take up heme, suggesting that *V. anguillarum* may harbor two TonB systems (M. Stork, M. Di Lorenzo, S. Mouriño, C. R. Osorio, M. L. Lemos, and J. H. Crosa, unpublished data).

The goal of the present study was to identify and characterize the genes involved in heme transport in *V. anguillarum*. Sequences of the heme uptake cluster genes were determined and analyzed, and deletion mutants were constructed and tested for the ability to grow with hemin or hemoglobin as the sole iron source. Complementation of *E. coli* mutants with *V. anguillarum*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
V. anguillarum H-775-3	Serotype O1 plasmidless avirulent strain, deficient in anguibactin biosynthesis, derived from strain 775 by curing of pJM1	J. H. Crosa
E. coli		
DH5α	Cloning strain	Laboratory stock
SM17-1-λpir	Cloning strain	Laboratory stock
EB53	*aroB hemA*	6
101ESD	HB101 derivative, deficient in enterobactin biosynthesis Δ(*entC-entA*)	J. H. Crosa
Plasmids		
pGEMT-Easy	PCR cloning vector, Ap^r	Promega
pWKS30	Low-copy-number plasmid vector, Ap^r	41
pACYC177	Cloning vector, Ap^r Km^r	33
pACYC184	Cloning vector, Ap^r Cm^r	33
pVK100	Mobilizable cloning vector, Tc^r Km^r	15
pRK2013	Mobilizing plasmid for triparental mating, Km^r	5
pKEK229	Suicide vector derived from pCVD442, Ap^r	3
pNidKan	pKEK229 with a 1.5-kb PstI-PstI fragment containing the Km cassette from pKANπ	This study
pML1	21-kb fragment from a gene library of strain H775-3 cloned into pVK100, Tc^r	25
pCAR121	ApaL1 fragment of pML1 containing *huvA* gene cloned into pACYC177; Km^r	This study
pCAR126	ApaL1 fragment of pML1 containing *huvX*, *tonB-exbBD*, and *huvB* genes and partial *huvZ* and *huvC* genes cloned into pACYC177, Km^r	This study
pCAR115	PCR-amplified *huvA* gene cloned into pWKS30, Ap^r	This study
pCAR179	*huvZ* gene cloned in pWKS30, Ap^r	This study
pCAR181	*huvX* gene cloned in pWKS30, Ap^r	This study
pSML11	EcoRV fragment of pML1 containing complete *huvZX* genes cloned into pWKS30	This study
pSML23	9-kb PCR fragment encoding the heme uptake system of *V. anguillarum* H-775-3 cloned into pWKS30, Ap^r	This study
pSML32	PstI-EcoRI fragment of pSML23 containing *huvBCD* genes cloned into pWKS30, Ap^r	This study
pSML33	SacI-ApaI fragment of pSML23 containing *tonB-exbBD* and *huvBCD* genes cloned into pWKS30, Ap^r	This study
pSML34	SacI-HindIII fragment of pML1 containing *tonB-exbBD* genes cloned into pWKS30, Ap^r	This study

genes for restoration of heme utilization as an iron and porphyrin source was also evaluated.

MATERIALS AND METHODS

Plasmids, bacteria, and media. Plasmids and bacterial strains used in this study are listed in Table 1. *V. anguillarum* cells were routinely grown at 25°C in tryptic soy agar (Difco) supplemented with 1% NaCl (TSA-1), as well as in M9 minimal medium (27) supplemented with 0.2% Casamino Acids (Difco) (CM9). *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth, LB agar, or CM9 supplemented with antibiotics when appropriate. Strain EB53 *aroB hemA* and its derivatives were routinely grown in LB medium supplemented with 2 μg of 5-aminolevulinic acid (ALA; Sigma) ml^{-1}. All strains were stored frozen at −80°C in LB broth with 20% glycerol. Antibiotics were used at the following final concentrations: tetracycline hydrochloride at 15 μg ml^{-1}, kanamycin at 25 μg ml^{-1}, and ampicillin sodium salt at 50 μg ml^{-1}. 2,2′-Dipyridyl (Sigma), used to chelate nonheme iron, was prepared at 10 mM in ultrapure water (milli-Q; Millipore). Bovine hemin (Sigma) was dissolved at 5 mM in 10 mM NaOH. Bovine hemoglobin (Sigma) was dissolved at 1 mM in ultrapure water.

Recombinant DNA techniques. Recombinant DNA methods including restriction-enzyme digestions, ligation reactions, agarose gel electrophoresis, and plasmid analysis were performed following standard protocols (34). Chromosomal DNA was isolated by using the Easy-DNA kit (Invitrogen). Plasmid purification and elution of DNA fragments from agarose gels were performed with kits from QIAGEN. Southern blot analyses were performed with Hybond-N+ membranes (Amersham Biosciences), using the ECL Direct Nucleic Acid Labeling and Detection system (Amersham Biosciences), following the manufacturer's instructions. *E. coli* strains were transformed by a standard calcium chloride method (34). Triparental mating for transfer of cosmid pML1 into *E. coli* EB53 was performed as described previously (8). *E. coli* strain HB101 harboring the helper plasmid pRK2013 was used as a mobilizing strain (5).

DNA sequencing and data analysis. DNA sequences were determined by the dideoxy-chain termination method by using the Big Dye Terminator v3.0 DNA sequencing kit (Applied Biosystems) on an automated sequencer, ABI 377 (Applied Biosystems). Restriction maps were generated and DNA translation was performed with the BioEdit Sequence Alignment Editor (11). Homologies of the deduced amino acid sequences were determined by consulting the EMBL and SWALL databases, using the FASTA3 and BLAST algorithms, at the European Bioinformatics Institute website.

Construction of chromosomal mutations. Gene deletions in *V. anguillarum* H-775-3 were constructed by using PCR amplifications of two fragments of each gene, which when ligated together would result in an in-frame (nonpolar) deletion. Amplification was carried out using the Expand High Fidelity PCR system (Roche). The oligonucleotides used to amplify the carboxy- and amino-terminal fragments of each gene are listed in Table 2. Construction of an in-frame *huvAZXBCD tonB exbBD* mutant was accomplished by ligating the PCR products obtained with primer pairs HuvA.A-HuvA.B and HuvD.C2-HuvD.D. Allelic exchange was carried out using the suicide vector pNidKan. Plasmid pKEK229 (3) was cut with PstI, and a kanamycin resistance cassette (Genblock; Pharmacia) was inserted, producing pNidKan. As a pCVD442 derivative, pNidKan contains R6K *ori*, requiring the *pir* gene product for replication, and the *sacB* gene, conferring sucrose sensitivity. Construction of in-frame deletions of *huvX*, *huvZ*, *huvC*, *huvD*, *huvB*, *huvA* and deletion of the complete gene cluster occurred in several steps. The PCR-amplified carboxy-terminal gene fragments were ligated into pWKS30, and resulting plasmids were cut with appropriate enzymes and ligated to the amino-terminal PCR fragments of each corresponding gene. This process resulted in the formation of mutant alleles Δ*huvZ* (removes coding sequences for amino acids 76 to 126), Δ*huvX* (removes coding sequences for amino acids 90 to 144), Δ*huvC* (removes coding sequences for amino acids 19 to 291), Δ*huvB* (removes coding sequences for amino acids 18 to 259), Δ*huvD* (removes coding sequences for amino acids 183 to 345), and Δ*huvA* (removes coding sequences for amino acids 178 to 698) and the complete gene cluster deletion mutant Δ*huv* (removes coding sequences encompassing amino acid 178 encoded by *huvA* and amino acid 345 encoded by *huvD*). Each deleted allele cloned in pWKS30 was digested with NotI and SalI and ligated into the NotI/SalI sites of the suicide vector pNidKan. The resulting plasmids were mated from *E. coli* S17-1-λ*pir* into *V. anguillarum* H-775-3, and transformants with the plasmid

TABLE 2. Oligonucleotides used in this study

Amplified region	Primer	Nucleotide sequence[a]
Mutant constructions		
huvA, amino terminal	HuvA.A	5′-GCTCTAGAAAGCTTTATCAACAATTGATT-3′
	HuvA.B	5′-TCCCCCGGGGGAAAGCTTTGCTTGTCCGCC-3′
huvA, carboxy terminal	HuvA.C	5′-TCCCCCGGGAACGACTACTCTCAAGCTGAG-3′
	HuvA.D	5′-GCGAATTCTGGGTAAAGAGGGCGAGTTAC-3′
huvZ, amino terminal	HuvZ.C	5′-GCGGATCCGACGTGCGGGTTCACCTCCAA-3′
	HuvZ.D	5′-GCGAATTCTATGATTTTACGAATTTAAAA-3′
huvZ, carboxy terminal	HuvZ.A	5′-GCTCTAGAGCTTCCGTGCCCCTAACTTTA-3′
	HuvZ.B	5′-GCGGATCCGACGGCTTAAGCCAGCTGCAA-3′
huvX, amino terminal	HuvX.A	5′-GCTCTAGATGTCGATATCACCGCTTACTT-3′
	HuvX.B	5′-GCGGATCCTTTATGGGACGTGAAAGTCAT-3′
huvX, carboxy terminal	HuvX.C	5′-GCGGATCCCATTCCCCAATCCGCTAATGA-3′
	HuvX.D	5′-GCAAGCTTGCATCGACTAATTTCGGTTGA-3′
huvB, amino terminal	HuvB.A	5′-GCTCTAGACAAGCGCCCATTGCTTTATAA-3′
	HuvB.B	5′-GCGGATCCGTCATTGGAGTGCAGCACGAG-3′
huvB, carboxy terminal	HuvB.C	5′-GCGGATCCGCTCTCGTCGGCGGGTTAGGC-3′
	HuvB.D	5′-GCGAATTCGTGAGTAAGAGTGCACCAAGT-3′
huvC, amino terminal	HuvC.A	5′-GCTCTAGACACGCTTTTAAAAACCGTATG-3′
	HuvC.B	5′-GCGGATCCGTCATTGGAGTGCAGCACGAG-3′
huvC, carboxy terminal	HuvC.C	5′-GCGGATCCATTGTGACTGCGCTGGTGGGT-3′
	HuvC.D	5′-GCGAATTCGCATTATAGCGAAGGGACGCG-3′
huvD, amino terminal	HuvD.A	5′-GCTCTAGACTTGTTGGTCGGCGCAATTTT-3′
	HuvD.B	5′-GCGGATCCCTTTGCTAACTCATTCGGTTG-3′
huvD, carboxy terminal	HuvD.C	5′-GCGGATCCTCGTCGCTTGTCGCCGCACAT-3′
	HuvD.D	5′-GCGAATTCGCTCGATCAGTGATGCGGTAT-3′
huvAZXBCD tonB exbBD, carboxy terminal	HuvD.C2	5′-TCCCCCGGGTCGTCGCTTGTCGCCGCACAT-3′
Gene amplification		
huvA	huvA-5′	5′-GCGAATTCTGGTGACAGCAGCGGCATCC-3′
	huvA-Eco-3′	5′-GCGAATTCAGGAGTTCATGGAACAACAAAGCC-3′
huvX	VA-huvZ-D	5′-GCGAATTCTATGATTTTACGAATTTAAAA-3′
	VA-HutZ-1	5′-CTCACATTCGGACGGCCTTCA-3′
huvZ ΔhuvX	PX3	5′-GCCTTTTCTTACAATGTCC-3′
	VA-TonB-4	5′-TGGTTTGGGCTTGTTGGCTC-3′

[a] Restriction sites for XbaI, BamHI, HindIII, EcoRI, and SmaI are underlined.

integrated into the chromosome by homologous recombination were selected on agar medium containing kanamycin and ampicillin. A second recombination event was obtained by selecting for sucrose resistance (10% wt/vol) and resistance to the specific antibiotic for the recipient strain (ampicillin). This led to obtention of *V. anguillarum* Δ*huvZ*, Δ*huvX*, Δ*huvC*, Δ*huvB*, Δ*huvD*, Δ*huvA*, and Δ*huv* mutant strains. Southern blot hybridization analysis was used to verify allelic exchange of the parental gene. In addition, for each mutant strain, the region involved in the deletion construction was PCR amplified and sequenced to ensure that the constructs were nonpolar (data not shown).

Subcloning of pML1 and construction of plasmids carrying *V. anguillarum* genes. Oligonucleotides used to amplify genes of the *V. anguillarum* heme uptake system are listed in Table 2. Cosmid pML1 was cut with ApaLI, and two fragments were subcloned in pACYC177, yielding pCAR121 (containing *huvA*) and pCAR126 (containing *huvX*, *tonB-exbBD*, *huvB*, and partial *huvZC* genes). An EcoRV fragment containing complete *huvZX* genes was cloned in pWKS30 to yield pSML11. A SacI-HindIII fragment including complete *tonB-exbBD* genes was cloned in pWKS30 to yield pSML34.

The complete nine-gene heme uptake cluster was PCR amplified with primers huvA-5′ and HuvC.D. The PCR product was cut with EcoRI and cloned in pWKS30 to produce pSML23. A PstI-EcoRI fragment of pSML23 containing *huvBCD* genes, and a SacI-ApaI fragment, including the *tonB* system plus *huvBCD* genes, were subcloned in pWKS30 to yield pSML32 and pSML33, respectively (in cases where a unique promoter exists upstream of *tonB* from which the six downstream genes are transcribed, *huvBCD* genes cloned in pSML32 will not be expressed).

The complete *huvA* gene including upstream and downstream DNA was amplified with primers huvA-5′ and huvA-Eco-3′. The PCR product was cut with EcoRI and cloned in pWKS30 to yield pCAR115. The complete *huvX* gene including upstream DNA was amplified with primers VA-huvZ-D and VA-HutZ-1. The PCR product was cloned in pGEMT-Easy, and the insert was cut with EcoRI and subcloned in pWKS30 to yield pCAR181. The complete *huvZ* gene including the putative *huvX* promoter and in-frame-deleted *huvX* open reading frame (ORF) was amplified from *V. anguillarum* Δ*huvX* with primers PX3 and VA-TonB-4 (this allows the cloning of *huvZ* independently of *huvX* while still maintaining the putative *huvX* promoter, from which it is feasible that transcription of *huvZ* may occur). The PCR product was cloned in pGEMT-Easy, and the insert was cut with EcoRI and cloned in pWKS30 to yield pCAR179. All the PCR-amplified *V. anguillarum* genes to be used in complementation assays were obtained with the Expand High Fidelity PCR system (Roche) and further DNA sequenced to ensure that no PCR errors were artificially introduced.

Hemin and hemoglobin utilization assays. To test the ability of *V. anguillarum* mutants to utilize hemin or hemoglobin as an iron source, overnight cultures of the parental strain, *V. anguillarum* H-775-3, and the mutant strains were adjusted to the same optical density and diluted 1:100 in fresh CM9 broth containing the iron source (hemin, 10 μM; or hemoglobin, 1 μM) with or without the iron chelator 2,2′-dipyridyl at 100 μM. Cultures were shaken at 25°C, and absorbance at 600 nm was monitored at 1-h intervals over 12 h.

Complementation experiments. To test which genes of the cluster were essential for the utilization of hemin and hemoglobin as iron and porphyrin sources, *E. coli* strain EB53 *aroB hemA* and strain 101ESD Δ*(entC-entA)* were transformed with several plasmids. One-hundred-microliter portions of overnight cultures were added to 3 ml of molten soft nutrient broth (NB) or CM9 and plated onto appropriately prepared NB, CM9, or CM9 supplemented with 100 μM 2,2′-dipyridyl plates. Sterile filter paper disks were loaded with 20 μl of 5 and 0.05 mM hemin and 1 mM hemoglobin. Disks spotted with 20 μl of 2 mg-ml^{-1} ALA or 5 mM $FeSO_4$ were included as positive controls for utilization of porphyrin and iron sources, respectively. Results were annotated as positive or negative after 24 h of incubation.

Nucleotide sequence accession number. The EMBL accession number for the sequence described in this article is AJ496544.

RESULTS

Nucleotide sequence analysis of *V. anguillarum* heme utilization genes. The genetic organization of the *V. anguillarum*

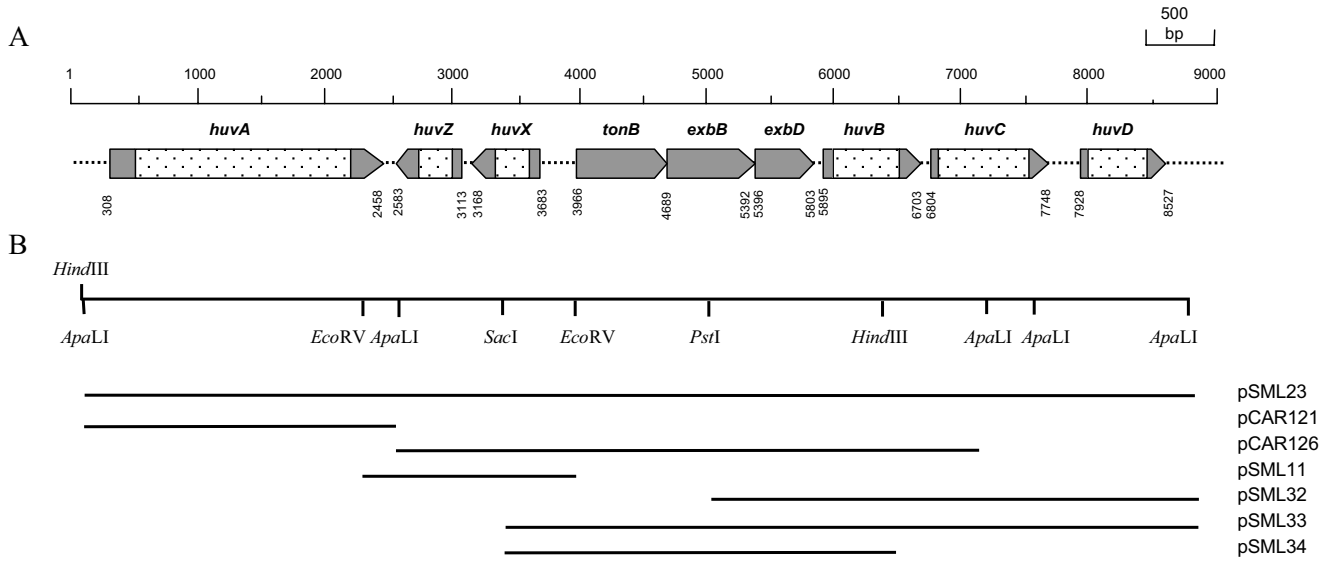

FIG. 1. (A) Physical map of the heme uptake gene cluster of *V. anguillarum* and mutant allele construction. ORFs are depicted as arrows, which indicate the direction of transcription, and the vertical numbers show the start and the end points of each gene. Deleted regions within each gene are shown as stippled boxes. (B) Relevant plasmid derivatives of the heme uptake cluster and restriction sites used in subcloning procedures.

heme utilization gene cluster was determined by partial DNA sequence analysis of pML1, a cosmid which enabled *E. coli* 101ESD to utilize heme as an iron source and which contains the heme receptor gene, *huvA* (25). The two strands of a DNA region downstream of *huvA* spanning ca. 6,000 bp were sequenced, and eight closely linked ORFs were identified (Fig. 1). The predicted products display significant similarity to components of other heme uptake systems in *Vibrio* and *Plesiomonas* species (Table 3).

A gene which we termed *huvZ* was found 125 bp downstream of the *huvA* stop codon and is transcribed from the opposite strand. *huvZ* encodes a 176-amino-acid protein with homology to proteins linked to heme transport systems (Table 3). The function of HuvZ homologues remains uncharacterized, though some observations suggest that *P. shigelloides* HugZ could be involved in preventing heme toxicity (14). Interestingly, database comparisons evidence that HuvZ has homology to proteins containing a flavin mononucleotide-binding split barrel (data not shown). This homology suggests that this protein may play a role in processes of electron transfer, with the heme group being involved.

The next ORF in the cluster corresponds to the *huvX* gene, located 55 nucleotides upstream of the *huvZ* start codon, and codes for a predicted protein of 171 amino acids. The highest similarities were to proteins linked to heme transport systems of gram-negative bacteria (Table 3). None of the HuvX homologues has a known function, but it has been suggested that *P. shigelloides* HugX could be involved in preventing heme toxicity (14).

TABLE 3. Proteins with homology to products of *V. anguillarum huvZ*, *huvX*, and *huvBCD* genes

V. anguillarum protein	Homologue	EMBL accession no.	Amino acid identity (%)	Amino acid similarity (%)
HuvZ	*V. cholerae* hypothetical protein	Q9KL41	82	93
	V. parahaemolyticus hypothetical protein	Q87J24	82	92
	V. vulnificus hypothetical protein	Q8D3S0	81	90
	P. shigelloides HugZ	Q93ST0	64	78
HuvX	*V. cholerae* hypothetical protein	Q9KL40	78	91
	V. parahaemolyticus hypothetical protein	Q87J25	74	88
	V. vulnificus hypothetical protein	Q8D3S1	71	87
	P. shigelloides HugX	Q93SS9	51	70
HuvB	*V. parahaemolyticus* HutB	Q87J30	68	82
	V. cholerae HutB	Q9KL36	66	81
	V. vulnificus heme transporter	Q8D3S6	64	80
	P. shigelloides HugB	Q93SS3	44	64
HuvC	*V. cholerae* HutC	Q9KL35	75	90
	V. parahaemolyticus HutC	Q87J31	74	87
	V. vulnificus heme transporter	Q8D3S7	75	85
	P. shigelloides HugC	Q93SS2	61	78
HuvD	*V. cholerae* HutD	O52047	70	83
	V. parahaemolyticus HutD	Q87J32	71	86
	V. vulnificus heme transporter	Q8D3S8	60	77
	P. shigelloides HugD	Q93SS1	48	66

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V.anguillarum       LSTTAAIQASNISVTFGHRTILDKIDIEIFSGQVTALLGPNGAGKSTLLKILSGEISSTG 60
V.cholerae          M-QTIAIQGRDLCVTYGSRQVLDHVDITLRCGEVAALLGPNGAGKSTLLKLLCGEMSGAG 59
V.parahaemolyticus  M-------------KYGHRLVLDDISIDIRAGEVTALLGPNGAGKSTLLKLLCGEVPSHN 47
V.vulnificus        MKKPVVLRGQNLSLQFASRQVLKQIDVAFCAGEVVALLGPNGAGKSSLLKLLSGEITSSQ 60
                                 : :. * :*..:.: : .*:*.***********:***:*.**:..

V.anguillarum       KMAYFGVPQALWQPNELAKHLAILPQQSTLSFPFIAQEVVELGALPLNLSHQQVSEVALH 120
V.cholerae          KLDYFGVPASQWPAEKLANHLGILPQQSSLTFPFTAQEVVELGAIPLNLPRKEVERVARH 119
V.parahaemolyticus  EIDYFGEPKEAWKPEEIAKHLAMLPQHSTLTFPFLAREVVELGAIPLSLSNKETTELALH 107
V.vulnificus        SIEYFGKAAKSWRSAALSRHLGLLPQSSSLTFPFLAREVVELGAIPLALSQAEVKTIAEK 120
                    .: *** .   * .  ::.**.:*** *:*:*** *:*******:** *.. :.  :* :

V.anguillarum       YMQQTDISDRANNLYPALSGGEKQRLHLARVLTQLHHSGDKKILMLDEPTSALDLAHQHN 180
V.cholerae          YMLKTDVLHLAASLYPSLSGGEKQRLHLARVLTQLHQAGQQRILMLDEPTSALDLAHQHN 179
V.parahaemolyticus  YMQKTDVLHLAESLYPALSGGEKQRLHLARVLTQLHQSGDKKILMLDEPTSALDLAHQHN 167
V.vulnificus        YMAITDVVHLADSLYPALSGGEKQRLHFARVLTQLDQSGDKKILMLDEPTSALDLAHQHN 180
                    **  **: . * .***:**********:*******.::*:::****************

V.anguillarum       TLRIARSLAHQEQCAVVVVLHDLNLAAQYADRMVMLHNGKLVCDAPPWEALNAERIEQVY 240
V.cholerae          TLQLARQLADEEQCAVVVVLHDLNLAAQYSDRLILLHQGKIVCDAAPWQALTAERIEQVY 239
V.parahaemolyticus  TLKIAREAAKAQNAAVVVVLHDLNLASQYADRLVLLHNGKLVCDDNPWQALTPERIEQVY 227
V.vulnificus        TLRVAKQFAKEQNACVIVVLHDLNLAAQYADRMVILHRGEIVVDACPEEALTPEIIDAVY 240
                    **::*:. *. ::..*:*********:**:**:::**.*::* *  * :**..* *: **

V.anguillarum       GYSSLVAAHPTMDFPMVYPI- 260
V.cholerae          GYQALVAAHPTRDFPMVYPA- 259
V.parahaemolyticus  GYRSIVTKHPTLDFPQVHAAA 248
V.vulnificus        GYKAMIGRHPTLGFPLVQPAA 261
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FIG. 2. Alignment of the *V. anguillarum* HuvD ORF with homologues of other *Vibrio* species. Predicted amino acid sequence starting from a putative TTG start codon is shown in italics. Conserved GPNGAGKS, LMLDE, and LSGGE domains are shown in bold. Asterisks denote amino acids conserved in all the compared sequences.

Six additional genes are transcribed from the same strand as *huvA*. The three ORFs located adjacent to *huvX* encode the three proteins TonB, ExbB, and ExbD. The remaining three ORFs located downstream of *exbD* in this cluster code for proteins which show characteristics of heme transport proteins (Table 3). The predicted start codon for the seventh ORF of the cluster, *huvB*, is 51 nucleotides downstream from the stop codon of *exbD*. It encodes a predicted 282-amino-acid protein with homology to putative periplasmic heme-binding proteins. These proteins are believed to be involved in the transport of heme across the periplasm from the receptor to the ABC transporter located in the inner membrane.

The eighth ORF of the cluster encodes a 314-amino-acid protein which we termed HuvC, which has homology to members of a family of ABC-type permease proteins involved in the uptake of iron (Table 3). The last ORF of the cluster encoded a protein termed HuvD, which showed homology to the ATP-binding protein component of permeases involved in heme transport (Table 3). The first putative ATG of the *V. anguillarum* HuvD ORF determines a protein of 199 amino acids, which is shorter than homologues such as *V. cholerae* HutD or *P. shigelloides* HugD. Two TTG triplets, which is an unusual start codon in eubacteria (9), are located in frame upstream of the candidate ATG start codon and can be considered putative start codons for *V. anguillarum* HuvD. The amino acid sequence translated from any of these TTG triplets show high similarity to the N-terminal amino acids of *V. cholerae* HutD. Considering one of these two in-frame TTG triplets as the start codon, the larger *V. anguillarum* predicted HuvD ORF encodes a protein with features common to ABC transporter ATPases: the walkerA nucleotide-binding consensus motif GPNGAGKS (located 42 bp upstream of the first ATG codon) and the walkerB motif LMLDE at positions 103 to 107 downstream of the putative ATG start. These two sites are part of a highly conserved ATP-binding motif that constitute an ATP-binding pocket (40). In addition, an ABC transporter signature motif, LSGGE, is found at positions 77 to 81 of HuvD (Fig. 2). The presence of these features suggests that *V. anguillarum* HuvD is the ATPase component of the heme ABC transporter.

Similarities with other heme uptake gene clusters. The spatial organization of heme uptake genes in the chromosome of *V. anguillarum* is similar to that described for other *Vibrio* species and for *P. shigelloides* (Fig. 3). This fact suggests that the genes of the heme transport cluster were acquired by horizontal gene transfer, simultaneously with the TonB genes. This may have represented a selective advantage, allowing efficient heme transport by the recipient strain. However, some differences in gene order are observed. Among the *Vibrio* species, *V. anguillarum* is unique in that the outer membrane receptor gene is linked to the rest of the heme transport genes. Similarly, upstream of the *huvX* homologue, all other *Vibrio* species, as well as *P. shigelloides*, contain a gene which is transcribed in the same direction as *huvX* and *huvZ* homologues and is absent in the *V. anguillarum* heme uptake cluster. This gene codes for a putative coproporphyrinogen oxidase (it has been named HutW, PhuW, or HugW), an enzyme that converts coproporphyrinogen III into protoporphyrin IX, one of the steps in the heme biosynthesis pathway (31).

Finally, a gene (*hupR*) coding for a member of the LysR family of transcriptional activators, which regulates expression

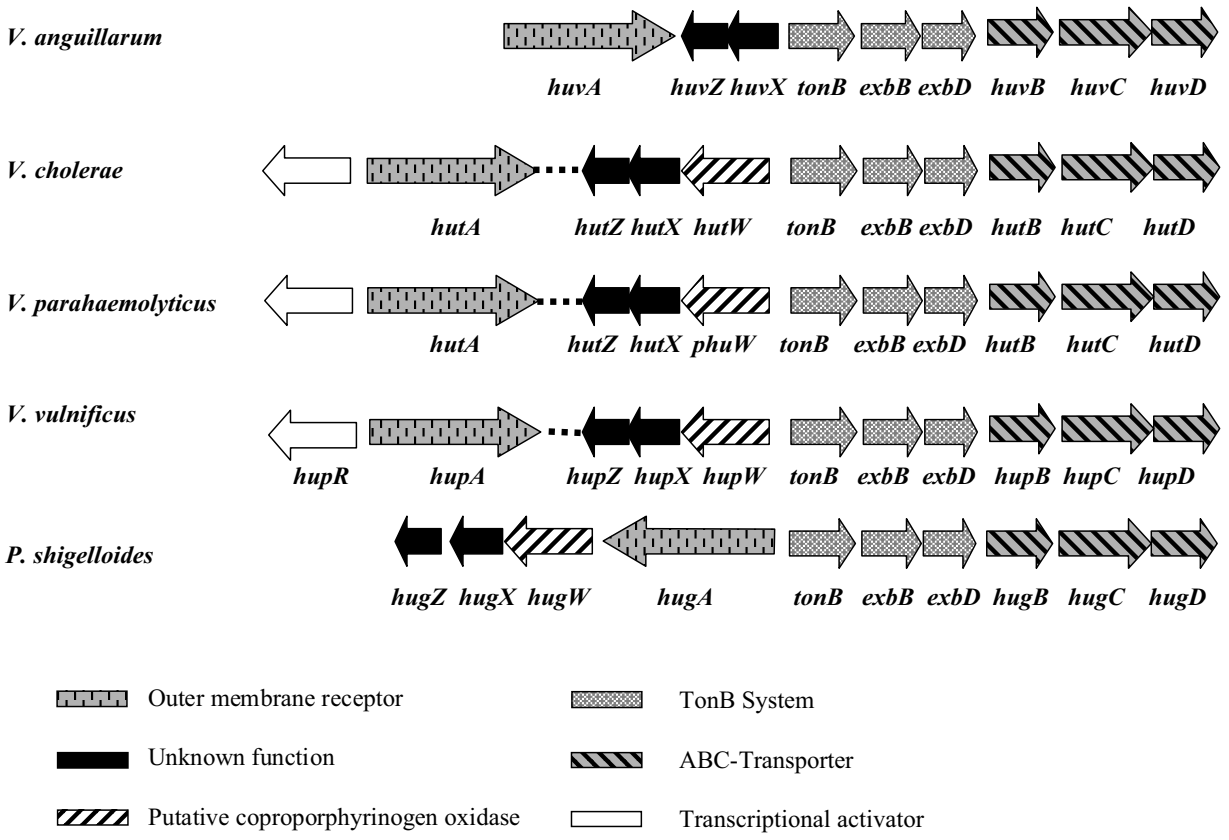

FIG. 3. Comparative chromosomal arrangement of heme uptake cluster genes in *Vibrio* species and *P. shigelloides*.

of the heme receptor, has been found in *V. vulnificus* (21). Homologues of this gene are encountered upstream of *V. parahaemolyticus hutA* (22) and *V. cholerae hutA* (12). However, the nucleotide sequence immediately upstream of *V. anguillarum huvA* does not code for an HupR homologue (data not shown).

Phenotypic analysis of heme uptake system chromosomal mutants with hemin or hemoglobin as the only iron source. In order to evaluate the importance of individual genes of the heme uptake system in the utilization of hemin or hemoglobin, individual in-frame deletions of *huvA*, *huvZ, huvX*, *huvB*, *huvC*, and *huvD* and an in-frame deletion of the complete nine-gene cluster were constructed. The H-775-3 strain, deficient in siderophore anguibactin biosynthesis, was chosen in order to eliminate background growth resulting from anguibactin-mediated iron uptake in iron-restricted media. Growth assays were carried out in triplicate, and results shown here are the means of three independent experiments. Strains were first assayed in an iron-sufficient medium with hemin (10 μM). No significant differences in growth rates were seen in iron-sufficient medium between mutant strains and *V. anguillarum* H-775-3 (Fig. 4A). The same mutants were then grown in an iron-restricted medium with the iron chelator 2,2′-dipyridyl added at a concentration of 100 μM and containing 10 μM hemin as the sole iron source. Under these conditions, no significant differences in growth were observed between the Δ*huvX* strain and the parental strain, *V. anguillarum* H-775-3, with the slight differences observed likely due to a lower initial inoculum. This demonstrates that this gene is not essential for the utilization of hemin as the sole iron source in *V. anguillarum*. By contrast, the rest of the assayed mutants were drastically affected in their ability to use hemin. Mutation of any *huvAZBCD* gene decreased bacterial growth to minimal levels (Fig. 4B). The Δ*huv* strain showed a reduction in growth with hemin as the sole iron source comparable to that observed in the single Δ*huvA*, *-Z*, *-B*, *-C*, and *-D* mutants (Fig. 4B). The same results were obtained for all the assayed strains when hemoglobin was used as the iron source instead of hemin (data not shown).

Utilization of heme compounds by *E. coli* EB53 and 101ESD complemented with *V. anguillarum* genes. The ability of *V. anguillarum* heme transport genes to allow the use of hemin or hemoglobin as porphyrin and iron sources was evaluated in *E. coli* EB53 (*aroB hemA*) and in *E. coli* 101 ESD [Δ*(entC-entA)*], respectively. The *aroB* and *entC-entA* mutations render *E. coli* unable to produce its own siderophore, enterochelin, and the *hemA* mutation disrupts synthesis of heme. Therefore, *E. coli* EB53 cannot grow unless supplied with the heme biosynthetic precursor ALA. EB53 could satisfy the porphyrin deficiency by utilizing exogenously supplied hemin, as long as a genetic system for hemin uptake is provided. Similarly, 101ESD cannot grow in the presence of iron chelators unless supplied with a utilizable source of iron.

Growth around hemin (0.05 and 5 mM) or hemoglobin (1 mM) disks on nutrient agar plates (NB) was used to assay the use of porphyrin sources by *E. coli* EB53. To test whether the introduction of the entire heme transport gene cluster would

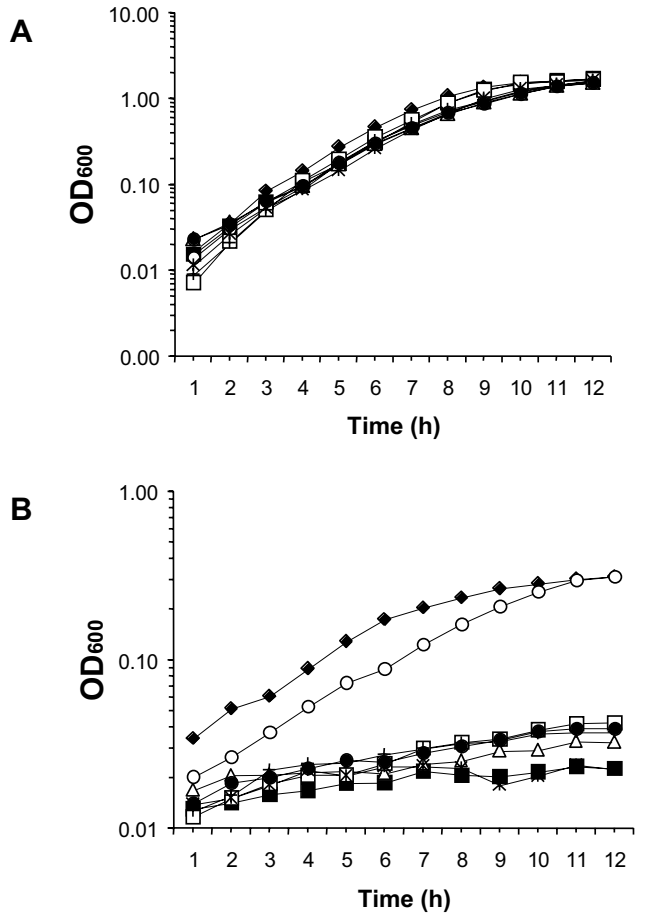

FIG. 4. Growth of *V. anguillarum* H-775-3 (◆) and Δ*huvA* (■), Δ*huvZ* (△), Δ*huvX* (○), Δ*huvB* (□), Δ*huvC* (●), Δ*huvD* (+), and Δ*huv* (∗) mutants in CM9 minimal medium. Growth was with hemin (10 μM) as the iron source without (A) and with (B) the free-iron chelator 2,2′-dipyridyl (100 μM). Results are expressed as the averages of three independent experiments. OD_{600}, optical density at 600 nm.

promote hemin and hemoglobin utilization as porphyrin sources, cosmid pML1, containing the entire heme transport region within a ca. 15-kb *V. anguillarum* H-775-3 genome fragment, was introduced into EB53 by triparental mating. This transformant utilized hemin and hemoglobin as porphyrin sources. A subclone of pML1 (pCAR121) containing only *huvA* proved to be sufficient to confer the utilization of hemin and hemoglobin as porphyrin sources upon *E. coli* EB53. This indicates that *E. coli* EB53 (a K-12 derivative) encodes all the additional functions necessary for transporting and utilizing heme as a porphyrin when an outer membrane heme receptor is provided. None of other plasmid combinations assayed, in which *huvA* was absent, could complement EB53 (data not shown).

To determine which genes are necessary for the utilization of hemin and hemoglobin as iron sources, *E. coli* 101ESD was complemented with *V. anguillarum* genes and tested on CM9 minimal medium plates supplemented with 100 μM 2,2′-dipyridyl and with hemin and hemoglobin supplied on paper disks. Results are summarized in Table 4. *E. coli* 101ESD transformed with plasmids containing *huvA* alone did not grow in this medium, indicating that other genes in addition to *huvA* are needed for the utilization of hemin or hemoglobin as an iron source. However, *E. coli* 101ESD transformed with cosmid pML1 utilized both compounds as iron sources. In order to determine the minimum genetic background necessary for utilization of heme as an iron source, different gene combinations were assayed.

Heme iron utilization did not occur when strain 101ESD/pCAR121 was transformed with pSML11 (*huvZX*) or with pCAR126 (*huvX tonB exbBD huvB*). The complete nine-gene cluster was PCR amplified, cloned in pWKS30 to yield pSML23, and transformed into 101ESD. This strain utilized hemin and hemoglobin as iron sources, indicating that this capacity is encoded within the nine-gene cluster described here and that this utilization is not due to extra genes present in pML1.

Complete *tonB exbBD huvBCD* genes cloned in pSML33 and transformed into strain 101ESD/pCAR121 allowed this strain to utilize hemin and hemoglobin as iron sources. However, 101ESD/pCAR121/pSML32 and 101ESD/pCAR121/pSML34 failed to grow with hemin or hemoglobin. These results together demonstrate that *tonB exbBD* genes in combination with *huvA* are not sufficient for heme iron utilization unless *huvBCD* genes are also provided. It is feasible that *huvBCD* genes are the crucial ones in combination with

TABLE 4. Utilization of hemin and hemoglobin as iron sources by *E. coli* 101ESD complemented with *V. anguillarum* heme uptake genes[a]

Strain	Gene(s) present	Utilization of:		
		Hemoglobin (0.1 mM)	Hemin (5 mM)[c]	$FeSO_4$
101ESD	None	−	−	+
101ESD/pCAR121	*huvA*	−	−	+
101ESD/pCAR126	*huvX tonB-exbBD-huvB*	−	−	+
101ESD/pML1	*huvAZX tonB-exbBD-huvBCD*	+	+	+
101ESD/pCAR115/pCAR126	*huvAX tonB-exbBD-huvB*	−	−	+
101ESD/pCAR121/pCAR179	*huvAZ*	−	−	+
101ESD/pSML23	*huvAZX tonB-exbBD-huvBCD*	+	+	+
101ESD/pCAR121/pCAR181	*huvAX*	−	−	+
101ESD/pCAR121/pSML11	*huvAZX*	−	−	+
101ESD/pCAR121/pSML32	*huvA-exbD-huvBCD*[b]	−	−	+
101ESD/pCAR121/pSML34	*huvA tonB-exbBD*	−	−	+
101ESD/pCAR121/pSML33	*huvA tonB-exbBD-huvBCD*	+	+	+

[a] Assay was conducted on CM9 plates supplemented with 100 μM 2,2′-dipyridyl.
[b] Genes *exbD-huvBCD* in pSML32 will not be expressed if they are transcribed from a promoter upstream of *tonB*.
[c] The same results were obtained when 0.05 mM hemin was used.

huvA to allow transport of heme compounds into the cytoplasm of *E. coli* and further utilization as iron sources. The finding that *tonB exbBD* genes need to be provided in the same plasmid together with downstream *huvBCD* genes in order to allow complementation may be explained by the existence of a single promoter upstream of *tonB*, as has been reported for *V. cholerae* (29).

DISCUSSION

Utilization of hemin and heme-containing proteins as iron sources has been reported for *V. anguillarum* (23, 24), but the molecular mechanism supporting heme uptake is unknown. In a recent work, the *V. anguillarum* outer membrane heme receptor gene *huvA* was characterized, and a *huvA* mutant obtained by chemical mutagenesis showed a reduction in virulence for fish (25). In the present study, we characterized a gene cluster in *V. anguillarum* that has similarities with heme iron assimilation systems found in other *Vibrio* species and *P. shigelloides*, both at the amino acid level and gene organization.

Homologues of *V. anguillarum* HuvZX proteins have been described, associated with the heme utilization systems of *V. cholerae*, *V. vulnificus*, *Vibrio parahaemolyticus*, and *P. shigelloides*, but to date their roles remain unascertained. In the present study we report the mutational analysis of *V. anguillarum huvZX* genes. Our results have shown that a *huvX* deletion mutant is able to use heme nearly as efficiently as the parental strain. This suggests that either *huvX* is not directly involved in the use of heme as an iron source or additional *V. anguillarum* genes may substitute for the function of *huvX*. However, deletion of *huvZ* drastically reduces the ability of the bacterium to grow with heme as the sole iron source, indicating that this gene is essential for heme iron utilization. A HuvZ-related activity may be present in *E. coli* 101ESD, since the heme uptake system of *V. anguillarum* can be easily reconstituted in 101ESD without HuvZ. However, the actual function of HuvZ remains unknown. A possibility would be that HuvZ is involved in removing iron from heme. It has been proposed that the oxidative cleavage of heme mediated by heme oxygenases is a mechanism for iron acquisition for some bacteria. Heme oxygenase genes have been described recently for *Neisseria meningitidis* (44), and *Corynebacterium diphtheriae* (42), but no significant homology exists between these described heme oxygenase genes and HuvZ. Recently, Wyckoff et al. (43) demonstrated that *hutZ* (homologous to *huvZ*) is also essential for heme iron utilization in *V. cholerae*. These authors could not demonstrate a heme oxygenase activity for HutZ, suggesting that it may act as a heme carrier or storage protein. Further studies are needed in order to ascertain the role played by HuvZ in the utilization of heme as an iron source.

V. anguillarum huvBCD genes are essential for heme uptake. Nonpolar deletions in any of these three genes drastically reduce the growth of *V. anguillarum* with hemin or hemoglobin as the sole iron source. In contrast, it has been reported that *V. cholerae hutB, hutC*, or *hutD* mutants still retain significant growth when hemin is used as the only iron source (29). Thus, it is possible that proteins of other transport systems of *V. cholerae* can substitute for the physiological role of either HutB, -C, or -D, while in *V. anguillarum* each one of these proteins is essential for heme uptake.

Complementation studies carried out with *E. coli* EB53 showed that the *V. anguillarum* outer membrane heme receptor HuvA is sufficient for utilization of heme compounds as porphyrin sources. Complementation of *E. coli* heme-deficient mutants with an outer membrane heme receptor provided in *trans* has been previously reported (36). Interestingly, the *P. shigelloides* heme receptor gene *hugA* could not complement an *E. coli* DHE-1 *hemA* mutant for the use of heme as a porphyrin source unless *P. shigelloides tonB-exbB-exbD* genes were also provided in *trans* (14).

In the use of heme compounds as an iron source, *huvA* plus *tonB exbBD huvBCD* are needed to reconstitute the *V. anguillarum* heme transport system in *E. coli* 101ESD (an HB101 derivative). Similarly, Occhino et al. (29) reported that utilization of hemin as an iron source can be reconstituted in *E. coli* 1017 (HB101 derivative, *ent*) with *V. cholerae hutA*, *tonB exbBD*, and *hutBCD* genes. In contrast, Henderson et al. (14) observed that a plasmid containing *hugWXZ* was necessary in addition to *hugA* and *tonB-exbBD* to reconstitute the *P. shigelloides* heme iron utilization system in *E. coli* 1017.

We hypothesize that the *V. anguillarum huvBCD* genes are crucial for heme iron utilization in *E. coli* 101ESD but the *tonB* and *exbBD* genes are not. Complementation of EB53 for heme utilization as a porphyrin source did not depend on the presence of the *V. anguillarum* TonB system in *trans*, which means that *huvA* alone is active in an *E. coli* background. We propose that *hutBCD* genes are transcribed from a promoter located upstream of the *tonB* gene, and thus actual complementation for heme iron utilization is achieved only when the six genes *tonB*, *exbBD*, and *huvBCD*, are provided together on a plasmid. It remains to be explained why heme utilization as a porphyrin source in *E. coli* EB53 does not depend on *huvBCD* genes while utilization as an iron source in *E. coli* 101ESD does require *huvBCD*. This could be due to a difference in strain background, as for example, the presence of an inner membrane transporter in EB53.

As reported by Stojiljkovic and Hantke (36), the *Y. enterocolitica* outer membrane heme receptor HemR alone is sufficient to complement *E. coli* for utilization of heme as a porphyrin source, and it is possible that hemin, once in the periplasm, can be incorporated into cytochromes located in the cytoplasmic membrane (10). However, utilization of heme compounds as iron sources would be feasible only as long as the heme compound is transported from the periplasmic space into the cytoplasm, where it is expected that additional proteins are implicated in degradation of the heme molecule and in the release of iron. As proposed by Stojiljkovic and Hantke (36), the difference between porphyrin and iron utilization from heme may be merely quantitative, being the amount of heme needed to satisfy the cell's requirements for iron, much larger than the amount necessary as a porphyrin source. This being the case, *E. coli* EB53 complemented with *V. anguillarum* HuvA could transport trace amounts of heme as a porphyrin source into the cytoplasm via nonspecific *E. coli* ABC transporters. However, heme iron utilization could be effective only as long as a specific ABC transporter (HuvBCD) is provided.

The gene coding for the *V. anguillarum* outer membrane heme receptor is linked to the rest of heme transport genes. Such a spatial organization is unusual in other species of the family *Vibrionaceae*, where the outer membrane receptor gene is separated from the rest of the transport genes by hundreds

of kilobases (1, 12, 22). Other differences in the *V. anguillarum* heme uptake cluster include the absence of a putative coproporphyrinogen oxidase gene and a gene for a LysR transcriptional activator homologue, which are present in *V. vulnificus*, *V. cholerae*, and *V. parahaemolyticus* (1, 12, 22). It is possible that the heme transport cluster originally included homologues of these genes, which eventually underwent a spatial reorganization in the genome of *V. anguillarum*.

In conclusion, we have shown in this study that the heme uptake cluster of *V. anguillarum* H-775-3 includes nine genes, five of which proved to be essential for utilization of heme as an iron source. The gene arrangement of the *V. anguillarum* heme uptake cluster has unique features which differentiate it from homologous clusters found in other gram-negative bacteria.

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