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Cloning and Characterization of an Outer Membrane Protein of *Vibrio vulnificus* Required for Heme Utilization: Regulation of Expression and Determination of the Gene Sequence

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***Vibrio vulnificus* is a halophilic, marine pathogen that has been associated with septicemia and serious wound infections in patients with iron overload and preexisting liver disease. For *V. vulnificus*, the ability to acquire iron from the host has been shown to correlate with virulence. *V. vulnificus* is able to use host iron sources such as hemoglobin and heme. We previously constructed a *fur* mutant of *V. vulnificus* which constitutively expresses at least two iron-regulated outer membrane proteins, of 72 and 77 kDa. The N-terminal amino acid sequence of the 77-kDa protein purified from the *V. vulnificus fur* mutant had 67% homology with the first 15 amino acids of the mature protein of the *Vibrio cholerae* heme receptor, HutA. In this report, we describe the cloning, DNA sequence, mutagenesis, and analysis of transcriptional regulation of the structural gene for HupA, the heme receptor of *V. vulnificus*. DNA sequencing of *hupA* demonstrated a single open reading frame of 712 amino acids that was 50% identical and 66% similar to the sequence of *V. cholerae* HutA and similar to those of other TonB-dependent outer membrane receptors. Primer extension analysis localized one promoter for the *V. vulnificus hupA* gene. Analysis of the promoter region of *V. vulnificus hupA* showed a sequence homologous to the consensus Fur box. Northern blot analysis showed that the transcript was strongly regulated by iron. An internal deletion in the *V. vulnificus hupA* gene, done by using marker exchange, resulted in the loss of expression of the 77-kDa protein and the loss of the ability to use hemin or hemoglobin as a source of iron. The *hupA* deletion mutant of *V. vulnificus* will be helpful in future studies of the role of heme iron in *V. vulnificus* pathogenesis.**

Vibrio vulnificus is a halophilic, marine pathogen that has been associated with primary septicemia and serious wound infections in immunocompromised individuals and patients who have cirrhosis, hemochromatosis, or alcoholism (5, 31, 32, 34). Primary septicemia is often acquired by eating shellfish, and wound infections are associated with exposure to seawater (52).

Iron is an important element essential to the growth of most bacteria. In the human body, most intracellular iron is found as hemoglobin, heme, ferritin, and hemosiderin. The trace quantities of iron present extracellularly are bound to high-affinity iron binding proteins such as transferrin and lactoferrin (4). Microorganisms have evolved various mechanisms for the acquisition of iron from the host; these mechanisms are closely linked to bacterial virulence. There are a number of virulence-associated determinants in pathogenic bacteria that are regulated by the iron status of the organisms, with increased gene expression occurring under conditions of low iron availability (1, 7, 9, 14). The expression of many of these iron-regulated genes are controlled at the transcriptional level by an iron-binding repressor protein called Fur (ferric uptake regulation) (3).

Iron seems to be particularly important in the pathogenesis of *V. vulnificus* infections. Wright et al. (55) directly correlated virulence of *V. vulnificus* with iron availability. They reported that the injection of iron into mice lowered the 50% lethal dose of a virulent strain of *V. vulnificus. V. vulnificus* is able to use host iron from sources such as hemoglobin, heme, and hemoglobin/haptoglobin complex (20). The lethality of intraperitoneal inocula of *V. vulnificus* is increased by concurrent injections of hemoglobin and hematin (20). However, the molecular mechanism of the utilization of hemoglobin and heme by *V. vulnificus* and importance in virulence are unknown.

The gene encoding the Fur protein of *V. vulnificus* was cloned, and a mutation was constructed in this gene by in vivo marker exchange (28). The *V. vulnificus fur* deletion mutant overexpressed at least two normally iron-regulated outer membrane proteins having apparent molecular masses of 72 to 77 kDa (28). The N-terminal amino acid sequence of the 77-kDa iron-regulated protein was determined, and the gene encoding this protein was subsequently cloned. In this communication, we report the cloning, mutagenesis, DNA sequence, and characterization of the gene encoding HupA, for heme uptake gene A, in *V. vulnificus*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Characteristics of the *V. vulnificus* and *Escherichia coli* strains and plasmids used in this study are described in Table 1.

Media. Strains were routinely grown in Luria broth (LB). All strains were maintained at −70°C in LB medium containing 15% glycerol. LB solidified with agar was used for high-iron solid medium. Two types of low-iron media were used: LB medium with or without the addition of the iron chelator 2,2′-dipyridyl (Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 0.2 mM and LB medium made iron deficient by the addition of 75 μg of ethylenediamine-di(*o*-hydroxyphenyl) acetic acid (EDDA), deferrated by the method of Rogers (41).

Ampicillin (100 μg/ml), kanamycin (45 μg/ml), polymyxin B (50 U/ml), tetracycline (15 μg/ml), or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; International Biotechnologies, Inc., New Haven, Conn.) (40 μg/ml) was added as appropriate.

Preparation and analysis of outer membrane proteins. Enriched outer membrane proteins from cells grown to late logarithmic phase in LB medium with and without added 2,2′-dipyridyl were prepared by using previously described procedures (19). The outer membrane proteins were separated on sodium dodecyl

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
V. vulnificus strains		
CML17	80363 Δ(*fur*)	28
MO6-24	Poly^r, opaque	55
CML49	MO6-24, Δ(*hupA*)	This study
E. coli strains		
DH5α	F$^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ*(argF-lacZYA)U169* (ϕ80Δ*lacZ* M15)λ$^-$	18
ABLE K	*lac*(LacZ$^-$) [Kan^r McrA$^-$ McrCB$^-$ McrA$^-$ McrF$^-$ Mrr$^-$ HsdR (r_K^- m_K^-)] [F′ *proAB lacI*^{q}ZΔ M15 Tn*10* (Tet^r)]	Stratagene
SY327λ*pir*	Δ(*lac pro*) *nalA recA56 araD argE*(Am) λ*pir* R6K	30
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir* R6K Km^r	30
Plasmids		
pUC19	Cloning vector; Ap^r	Laboratory stock
pBluescript SK$^-$	Phagemid derived from pUC19; Ap^r	Stratagene
pLAFR3	Cloning vector; Tc^r	48
pCML37	*V. vulnificus hupA* clone from *V. vulnificus* MO6-24 genomic library; 8-kbp chromosomal fragment in pBluescript SK$^-$; Ap^r	This study
pCML38	1.7-kbp *Hin*dIII *V. vulnificus hupA* clone in pBluescript SK$^-$; Ap^r	This study
pCML40	pBluescript SK$^-$ with *Hin*dIII insertion of *V. vulnificus hupA*, containing an internal 567-bp *Bgl*II-*Esp*I deletion; Ap^r	This study
pCVD442	Positive selection suicide vector, pGP704 with *sacB* gene inserted in multiple cloning site; Ap^r	10
pCML41	pCVD442 with *Hin*dIII insertion of *V. vulnificus hupA*, containing an internal 567-bp *Bgl*II-*Esp*I deletion; Ap^r	This study
pCML42	2.3-kbp *Nco*I-*Bam*HI *hupA* fragment and pBluescript cloned into the *Bam*HI site of pLAFR3; Tc^r Ap^r	This study

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Poly^r, polymyxin B resistance; Tc^r, tetracycline resistance.

sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) gels and were stained with Coomassie blue, as described previously (14).

N-terminal amino acid sequence analysis. For N-terminal amino acid analysis, outer membrane proteins from the *fur* mutant CML17 were electrophoresed by SDS-PAGE, electroblotted to polyvinylidene difluoride membranes (Bio-Rad, Richmond, Calif.), and stained with Ponceau S to localize the proteins. The 77-kDa protein was cut from the membrane, and the N-terminal amino acid sequence was determined by the Huntsman Cancer Institute peptide and DNA facility at the University of Utah. The N-terminal amino acid sequence was determined by standard Edman degradation on a model ABI 477A microsequencer (Applied Biosystems, Foster City, Calif.).

DNA manipulations and cloning. Standard methods were used for molecular biological techniques (42). Oligonucleotides were synthesized at the Huntsman Cancer Institute peptide and DNA facility. Oligonucleotides were radioactively labeled with T4 polynucleotide kinase, and plasmid DNA was radioactively labeled by random oligonucleotide-primed synthesis (Bethesda Research Laboratories Life Technologies, Gaithersburg, Md.).

The *hupA* gene was cloned by screening a recombinant lambda ZAPII phage genomic library of *V. vulnificus* MO6-24 constructed as described previously (29). After infection and plating of *E. coli* XL1 Blue, the resulting plaques were screened with the labeled oligonucleotide by using GeneScreen Plus colony-plaque membranes (DuPont, NEN Research Products) as described previously, except that low-stringency hybridization conditions were used (27). Purified phage isolated from the positive plaques were excised as Bluescript plasmids as described in the directions of the manufacturer (Stratagene, La Jolla, Calif.).

Restriction enzyme-digested genomic and plasmid DNA fragments were resolved through 1.0% agarose gels, and DNA was transferred to GeneScreen Plus membranes (DuPont, NEN Research Products) by the method of Southern (46). High-stringency hybridizations were performed at 42°C in a buffer containing 1 M NaCl, 1% SDS, and 50% formamide; the buffer used for low-stringency hybridizations contained 25% formamide instead of 50% formamide. After 6 to 24 h of hybridization, the membranes were washed as described in the manufacturer's recommendations and visualized by autoradiography.

DNA sequencing. The DNA sequence was determined by the dideoxy chain termination method of Sanger et al. (43) on double-stranded DNA plasmid templates by using a Sequenase kit from U.S. Biochemical Corporation, Cleveland, Ohio. Synthetic oligonucleotides used as primers for DNA sequencing were synthesized by the Huntsman Cancer Institute peptide and DNA facility, University of Utah.

Construction of *V. vulnificus hupA* mutant. A *hupA* deletion was constructed in *V. vulnificus* by in vivo marker exchange as described previously (6). Plasmid pCVD442 is a suicide vector containing the *sacB* gene, which allows positive selection with sucrose for the loss of plasmid sequences after homologous recombination into the chromosome (10). The 1.6-kb *Hin*dIII fragment of pCML37 was subcloned in pBluescript and designated pCML38; a 567-bp *Bgl*II-*Esp*I fragment internal to *V. vulnificus hupA* was deleted by digestion, Klenow treatment, and religation, and the deletion was confirmed by DNA sequencing to yield pCML40. The 1.1-kbp fragment of pCML40 was ligated into *Sac*I-*Sal*I-digested pCVD442, yielding pCML41. In vivo marker exchange was used to replace the chromosomal copy of *hupA* in *V. vulnificus* with the internal deleted copy in pCML41 without any remaining integrated plasmid sequences, as described previously (6, 10), to generate strain CML49. Construction of the deletion mutant was confirmed by Southern blot.

Utilization of iron sources. The utilization of iron sources by *V. vulnificus* was assayed by the procedure of Simpson and Oliver (44). Human holotransferrin (Sigma) solubilized in phosphate-buffered saline was determined to have an iron saturation of 99% by the Ferrozine assay for Fe (50) performed on a Hitachi 717 Automatic Analyzer (Boehringer Mannheim Corp., Indianapolis, Ind.). Hemin (Sigma) was solubilized in 10 mM NaOH, and hemoglobin was solubilized in phosphate-buffered saline. Vulnibactin, the catechol siderophore of *V. vulnificus* (37), was extracted from the culture supernatant of MO6-24 by the procedure of Griffiths et al. (17).

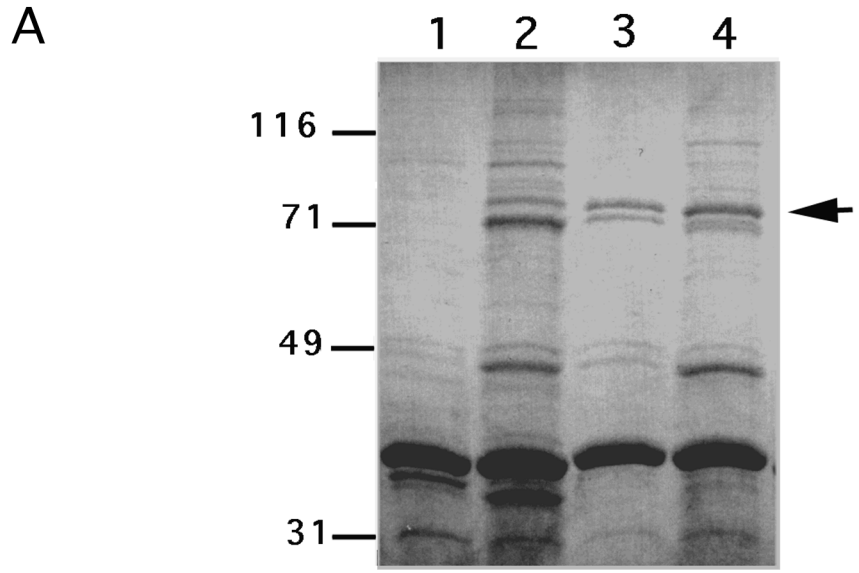
RNA analysis. RNAs from logarithmic-phase cultures grown under high-iron conditions (LB medium) and low-iron conditions (LB medium containing 2,2′-dipyridyl) were prepared by using Trizol reagent in accordance with the manufacturer's protocol (Bethesda Research Laboratories Life Technologies). A Northern (RNA) blot analysis was performed by using standard molecular biological techniques (42); equivalent amounts of RNA, as calculated from the optical density at 260 nm, were loaded into all of the lanes. The internal *Bgl*II-*Hin*dIII fragment of the *V. vulnificus hupA* gene was used as the probe. Primer extension was performed on RNAs from cultures grown under high-iron conditions and low-iron conditions with a Promega primer extension kit in accordance with the manufacturer's instructions (Promega, Madison, Wis.).

DNA and protein database searches. The National Center for Biotechnology Information services were used to consult the SwissPROT, GenBank, and EMBL databases with the BLAST algorithm (2, 12).

Nucleotide sequence accession number. The GenBank accession number for the sequence presented in this article is AF047484.

RESULTS

N-terminal amino acid sequence analysis of the 77-kDa iron-regulated outer membrane protein of *V. vulnificus*. Outer membrane protein preparations of a *V. vulnificus fur* mutant (CML17) constitutively express at least two outer membrane proteins, of 72 and 77 kDa, which are normally negatively regulated by iron in wild-type *V. vulnificus* (Fig. 1A). Compared with wild-type MO6-24, the *V. vulnificus fur* mutant (CML17) shows decreased expression of an approximately 33-



B

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CAA GAT GCT GGC TTA TTC GAT GAA GTA GTA GTA TCT GCA ACT CGC   Oligonucleotide
Q   D   A   G   L   F   D   E   V   V   V   S   A   T   R    N-terminal aa Sequence of 77 kDa protein
D   D   Y   A   S   F   D   E   V   V   V   S   T   T   R    V. cholerae HutA aa Sequence
GAT GAT TAT GCC TCA TTC GAT GAA GTA GTA GTA TCT ACA ACT CGC   V. cholerae hutA  DNA Sequence
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FIG. 1. (A) SDS-PAGE of outer membrane proteins. Lane 1, wild-type *V. vulnificus* grown in high-iron medium; lane 2, wild-type *V. vulnificus* grown in low-iron medium; lane 3, CML17 grown in high-iron medium; lane 4, CML17 grown in low-iron medium. The arrow indicates the position of the 77-kDa protein which was sequenced by Edman degradation. (B) Homology of N-terminal amino acid sequence with *V. cholerae* HutA sequence and synthesis of an oligonucleotide probe. The top single-letter-code sequence of amino acids (aa) is the N-terminal, 15-amino-acid sequence from the 77-kDa purified outer membrane protein from *V. vulnificus* CML17. The lower single-letter-code sequence of amino acids corresponds to the first 15 amino acids of *V. cholerae* mature HutA protein. The top nucleotide sequence is the sequence of the oligonucleotide used to probe the *V. vulnificus* genomic library. The bottom nucleotide sequence is the sequence of the nucleotides encoding this portion of the *V. cholerae* HutA amino acid sequence.

kDa protein. The N-terminal sequence of the 77-kDa protein isolated from the *fur* mutant yielded an N-terminal sequence of QDAGLFDEVVVSATR. A BLAST search (2) of the GenBank database found identity of this N-terminal sequence for 10 of the first 15 amino acids of the mature protein of the heme receptor of *Vibrio cholerae*, HutA (Fig. 1B). This homology suggested that the 77-kDa iron-regulated protein may be the *V. vulnificus* heme receptor.

Cloning of the gene encoding the 77-kDa protein of *V. vulnificus*. We initially synthesized a degenerate oligonucleotide on the basis of the N-terminal sequence of the 77-kDa protein, for use in hybridization. Attempts to clone the 77-kDa outer membrane protein by using a degenerate oligonucleotide based on the amino acid sequence FDEVVV (a region with identity to the *V. cholerae* HutA sequence) resulted in the isolation of several false-positive clones which had in common DNA sequences that encode DEV (data not shown). Subsequently, we synthesized a much longer oligonucleotide that was not degenerate. The sequence of the oligonucleotide was based on the N-terminal sequence of the 77-kDa protein, the frequency of codon usage for *V. vulnificus*, and the sequence of the *V. cholerae hutA* gene (Fig. 1B). We probed plaques from a *V. vulnificus* MO6-24 lambda ZAPII library with the oligonucleotide, which was end labeled with ^{32}P under low-stringency conditions (25% formamide, 42°C). Several plaques hybridized strongly with the oligonucleotide probe. Purified phages isolated from the positive plaques were excised as Bluescript plasmids. Two phagemids were successfully introduced into the *E. coli* ABLE K strain, which reduces the copy number of plasmids approximately 10-fold from the usual copy number. The *hupA* gene of *V. vulnificus* was localized by restriction mapping, using hybridization with the oligonucleotide and subsequent DNA sequencing. The *hupA* gene was contained in entirety in one clone, which was designated pCML37. The pCML37 plasmid was used in subsequent experiments. Subclones and a subclone containing an internal deletion of *hupA* are illustrated in Fig. 2.

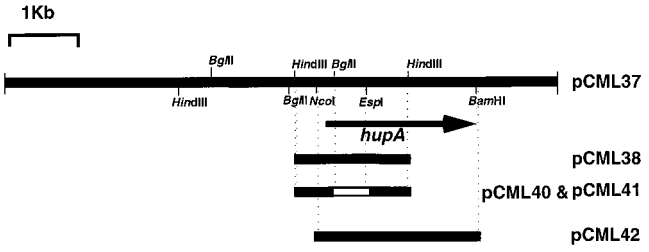

FIG. 2. Restriction map of *hupA* and flanking DNA. An approximately 8-kb fragment was cloned into lambda ZAPII and excised as a Bluescript plasmid to form pCML37. Plasmids pCML38 and pCML42 are subclones. Plasmids pCML40 and pCML41 contain the *Hin*dIII insert from pCML38 with an internal deletion of *hupA* DNA from the *Bgl*II site to the *Esp*I site, indicated by the open bar. In pCML40, the restriction fragment is cloned in pBluescript, and in pCML41, the same restriction fragment is cloned in pCVD442.

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NcoI                          Fur Box                    *
CCATGGTTAAAAATATTTTTACGAATGATAGTAATTATCATTAGCATCCAATATTATGACGACGATTATTCGCCTACTAT    80
                          -35                  -10
GGATTTAAGGTAGTAAAA  ATG TAC AAC AGA ACG TTC CTT TCA GCC TCA ATA CTA TTC GCT CTC    143
      SD            met tyr asn arg thr phe leu ser ala ser ile leu phe ala leu

GCT ACG CCA GTA CAA GCA CAA GAT GCT GGT TTA TTT GAT GAA GTT GTT GTT TCT GCA ACA    203
ala thr pro val gln ala gln asp ala gly leu phe asp glu val val val ser ala thr
                        ↑                                                 BglII
CGT ACG AAT CAA ACT ATG GAC TCA GTC GCT GCA TCA GTA ACT GTT ATC TCT GAC AAA GAT    263
arg thr asn gln thr met asp ser val ala ala ser val thr val ile ser asp lys asp
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FIG. 3. Partial nucleotide sequence of *V. vulnificus hupA* and its promoter region starting at the upstream *Nco*I site and ending at the *Bgl*II site within *hupA*. The locations of certain restriction sites are indicated. The deduced amino acid sequence of the first 55 amino acids of *V. vulnificus* HupA is shown below the *hupA* sequence. The approximate start site of transcription is indicated by an asterisk. The −35 region, the −10 region, and the Shine-Dalgarno sequence (SD) are underlined and labeled. The potential Fur box is labeled. A vertical arrow marks the signal peptidase cleavage site.

Nucleotide sequence analysis and predicted protein. The nucleotide sequence of *hupA* and its promoter region was determined. The upstream genetic region and a partial amino acid sequence of the N terminus are presented in Fig. 3. A 2,135-bp open reading frame begins 99 bp downstream of an *Nco*I restriction site. A putative Shine-Dalgarno sequence is located just upstream from the initiating methionine. A perfect inverted repeat, suggestive of a bidirectional transcriptional terminator, was found just beyond the termination codon (51). The precursor form of HupA contains a leader sequence of 21 amino acids, is 712 amino acids in length, and has a predicted molecular weight of 79,255. The mature protein has a predicted molecular weight of 76,958 which is in agreement with the observed mobility on SDS-PAGE gels. The calculated isoelectric point is 4.71. The average hydrophobicity of the mature protein is −0.55, indicating that the protein is hydrophilic in nature.

Primer extension analysis to localize the start site of *V. vulnificus hupA* transcription. Primer extension analysis of RNA from *V. vulnificus* MO6-24 grown under low- and high-iron conditions was done by using a synthetic oligonucleotide complementary to the DNA sequence just downstream of the initiating codon (Fig. 3) (bases 196 through 216). A single, strong primer extension product corresponding to base 63 of the sequence was identified only for RNA isolated from *V. vulnificus* MO6-24 grown under low-iron conditions (Fig. 4A and 3). Potential −35 and −10 boxes were identified upstream of the transcriptional start site. The putative Fur box is shown in Fig. 3, and the −35 box is contained within it. The *V. vulnificus hupA* Fur box has 15 of 19 nucleotides in common with the consensus sequence of the *E. coli* Fur box (9).

Northern blot analysis of the *hupA* transcript in *V. vulnificus*. Northern blot analysis was performed with RNA prepared from *V. vulnificus* grown in low- and high-iron media. The blot was probed with the *Bgl*II-*Hin*dIII fragment contained in the *V. vulnificus hupA* gene. One transcript of approximately 2,400 bases was observed only under low-iron conditions (Fig. 4B); this size is consistent with that predicted by the DNA sequence information. This indicates that *hupA* is monocistronic.

Homology of *V. vulnificus* HupA to *V. cholerae* HutA and other Ton B-dependent proteins. The amino acid sequences of *V. vulnificus* HupA and *V. cholerae* HutA have 50% homology and 66% similarity (22). The highest level of homology occurs at the amino-terminal and carboxy-terminal ends, with decreasing homology in the central portion of the protein. The nucleotide sequences of *V. vulnificus hupA* and *V. cholerae hutA* have 58.3% identity. *V. vulnificus* HupA also has signifi-

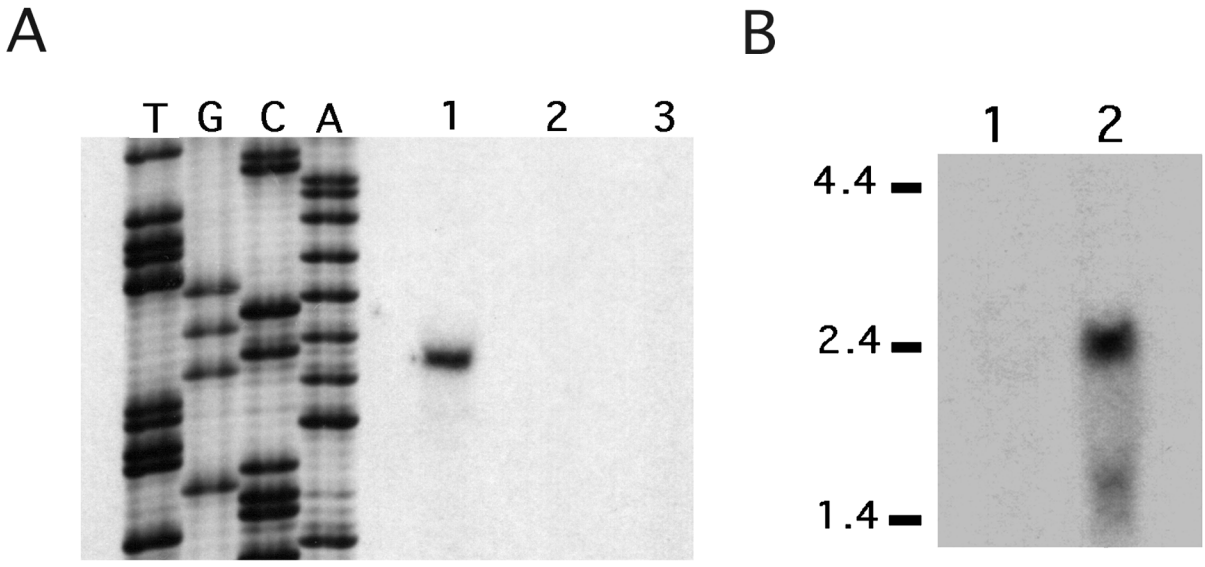

FIG. 4. (A) Primer extension analysis of RNA from *V. vulnificus hupA*. Lanes T, G, C, and A, lanes of the DNA sequencing ladder; lane 1, primer extension reaction mixture with *V. vulnificus* RNA prepared from a low-iron culture; lane 2, primer extension reaction mixture with *V. vulnificus* RNA from a high-iron culture; lane 3, primer extension reaction mixture without *V. vulnificus* RNA. (B) Northern blot analysis of RNA prepared from *V. vulnificus* after growth in high-iron medium (lane 1) and low-iron medium (lane 2) and probed with a *Bgl*II-*Hin*dIII fragment internal to *hupA*. The positions of RNA standards (in kilobases) are shown on the left.

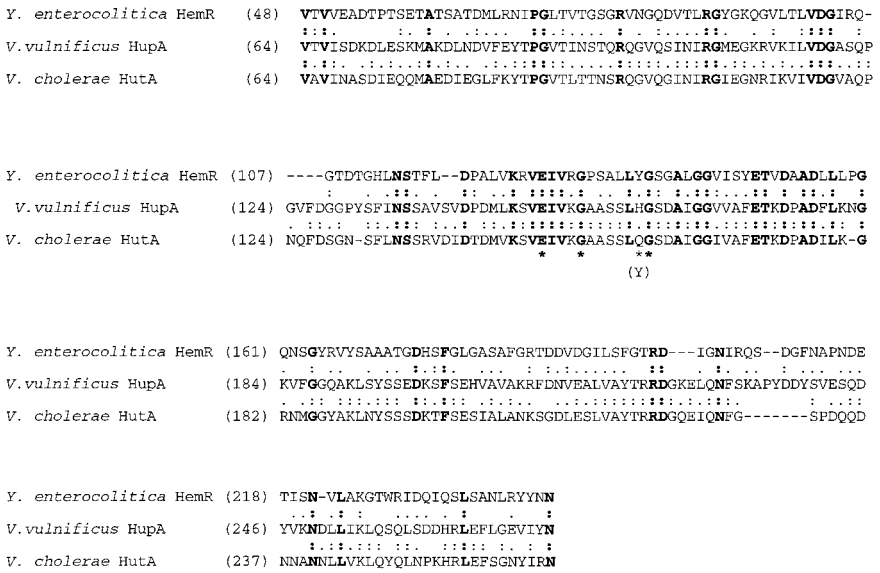

FIG. 5. Homology between HupA and the amino-terminal regions of *V. cholerae* HutA protein and *Y. enterocolitica* HemR protein. The numbers in parentheses indicate the position in the unprocessed protein of the first amino acid listed. Conserved amino acids between two proteins are indicated by colons, and substitutions of functionally similar amino acids are marked by periods. Letters in boldface type indicate amino acids conserved between all three proteins. The amino acids marked with an asterisk are those found by Nau and Konisky (35) to be conserved among TonB-dependent receptors in *E. coli*.

cant homology with a number of iron-regulated, TonB-dependent outer membrane proteins. Between 24 and 26% identity and 41 and 44% similarity were observed for *V. vulnificus* HupA and the following heme receptors: *Yersinia pestis* HmuR (GeneBank accession no. Q56989), *Yersinia enterocolitica* HemR (P31499), *Neisseria meningitidis* HmbR (U40860), *Shigella dysenteriae* ShuA (U64516), and *E. coli* ChuA (U67920). Additionally, the major iron-regulated outer membrane protein of *V. cholerae*, IrgA (P27772), had 25% identity and 41% similarity to *V. vulnificus* HupA. Figure 5 shows a comparison of regions near the amino termini of HemR of *Y. enterocolitica*, HupA of *V. vulnificus*, and HutA of *V. cholerae*.

When the TonB boxes of the *E. coli* vitamin B_{12} receptor BtuB and the *Y. enterocolitica* heme receptor HemR are compared with the homologous region of HupA of *V. vulnificus*, there is identity for four of six amino acids. One of the two most highly conserved amino acids among the TonB boxes is conserved in the *V. vulnificus* HupA sequence. The possible *V. vulnificus* TonB box is identical to the purported TonB box of *V. cholerae* HutA except for one amino acid difference.

Construction of a mutant of *V. vulnificus* with an internal deletion of *hupA* (strain CML49). To introduce an internal deletion of *hupA* into the chromosome of *V. vulnificus* by marker exchange, we constructed plasmid pCML41, a suicide vector containing a *Hin*dIII fragment of the *hupA* gene with a 567-bp internal deletion from the *Bgl*II site to the *Esp*I site within *hupA*. Plasmid pCML41 was transferred by conjugation into *V. vulnificus* MO6-24, with selection on medium containing ampicillin and polymyxin for the merodiploid state in which pCML41 had integrated into the chromosomal *hupA* by homologous recombination. The resulting merodiploid strain was grown without selection to late logarithmic phase, spread on plates containing 10% sucrose, and incubated overnight at 30°C. Sixteen of 200 sucrose-resistant colonies were sensitive to ampicillin, suggesting that vector sequences were lost.

(i) Verification of strain CML49 by Southern blot analysis. Of the 16 sucrose-resistant, ampicillin-sensitive colonies, one colony had a *hupA* gene sequence with the internal deletion. The genetic construction of strain CML49 was confirmed by Southern hybridization of *Hin*dIII-digested chromosomal DNA, probing with the cloned *Hin*dIII fragment of the *hupA* gene, and comparing the Southern blot results with the wild-type *V. vulnificus* DNA and the *hupA Hin*dIII fragment containing the internal deletion in pCML40. On a Southern blot, the wild-type *V. vulnificus* showed a 1.6-kbp hybridizing band and strain CML49 showed a 1.1-kbp hybridizing band (data not shown).

(ii) Verification of strain CML49 by analysis of iron-regulated outer membrane proteins. We additionally confirmed the *hupA* phenotype of CML49 by comparing the outer membrane proteins of wild-type *V. vulnificus* and strain CML49 after growth in low- and high-iron media (Fig. 6). In wild-type *V. vulnificus*, the two proteins with apparent molecular sizes of 72 and 77 kDa appear after growth under low-iron conditions. Mutant CML49 showed loss of expression of the 77-kDa iron-regulated protein.

Characterization of *hupA* mutant CML49. The *hupA* deletion mutant CML49 was tested for its ability to use hemin, hemoglobin, and other iron sources. As shown in Table 2, CML49 was unable to use hemin and hemoglobin as a source of iron.

Complementation of CML49 with pCML42. The entire *hupA* gene including the promoter from restriction site *Nco*I to *Bam*HI (2.4 kb) was subcloned into pLAFR3 (pCML42). When pCML42 was introduced into strain CML49, the ability to use hemin and hemoglobin was restored, indicating that *hupA* cloned on a plasmid could reconstitute heme utilization (Table 2). The mutant CML49 carrying the plasmid vector pLAFR3 did not differ in outer membrane protein expression from CML49 without vector (data not shown). Mutant CML49 containing pCML42 expressed an apparent 77-kDa outer membrane protein after growth in high-iron medium (Fig. 6). *V. vulnificus* CML49(pCML42) produced a large amount of protein of approximately 77 kDa in size under low-iron conditions, indicating iron-regulated synthesis of the 77-kDa HupA protein.

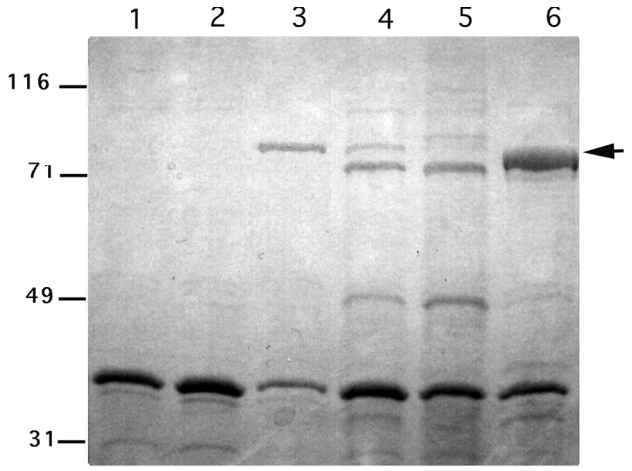

FIG. 6. SDS-PAGE of outer membrane proteins. Lane 1, wild-type *V. vulnificus* grown in high-iron medium; lane 2, strain CML49 grown in high-iron medium; lane 3, strain CML49(pCML42) grown in high-iron medium; lane 4, wild-type *V. vulnificus* grown in low-iron medium; lane 5, strain CML49 grown in low-iron medium; lane 6, strain CML49(pCML42) grown in low-iron medium. The numbers on the left indicate the positions of protein standards (in kilodaltons). The arrow indicates the position of HupA.

DISCUSSION

A number of adaptive responses have evolved in bacteria to allow competitive growth and survival in the host. The acquisition of iron is one of the most important of these adaptive responses for bacterial pathogenesis. Many iron transport systems in gram-negative bacteria involve iron-regulated outer membrane receptors. Most gram-negative bacteria have a Fur-like system for gene regulation in response to iron. Fur homologs have been identified in various gram-negative bacteria, including *Salmonella typhimurium*, *Serratia marcescens*, *V. cholerae*, *V. vulnificus*, *Y. pestis*, *N. meningitidis*, and *Pseudomonas aeruginosa* (11, 27, 28, 38, 39, 47, 54).

Numerous virulence factors have been suggested to be important in the pathogenesis of *V. vulnificus*, including a hemolysin-cytolysin (15, 16, 26), an elastolytic protease (24, 25), a polysaccharide capsule (56, 58), and a phospholipase (53). Many studies have also shown the importance of the ability of *V. vulnificus* to use host iron for the virulence of this organism (33, 44, 45, 49, 55). Recently, a vulnibactin (catechol siderophore) synthesis mutant of *V. vulnificus* was shown to have reduced virulence in an animal model (29). In *V. cholerae*, a number of iron-regulated genes have been characterized that are known to be regulated by Fur. These include genes for hemolysin production (40), genes encoding IrgA (14) (an iron-regulated outer membrane virulence determinant) and IrgB (13) (an iron-regulated positive transcriptional activator of IrgA), genes for siderophore synthesis (57) and transport (8), and the gene encoding HutA (23). Homologs for many of these genes probably exist in *V. vulnificus*, and some may be important in virulence. The promoter of the hemolysin-cytolysin gene of *V. vulnificus* may contain possible binding sites for the Fur protein, suggesting that it is regulated by iron and Fur. However, regulation of *V. vulnificus* genes by iron has not been studied in detail. In this report, we have described the cloning of the heme receptor of *V. vulnificus* and studied its regulation by iron. The high degree of homology between the proposed *hupA* Fur box and the *E. coli* consensus Fur box and the homology between the *V. vulnificus* and *E. coli* Fur proteins (28) predict that *V. vulnificus* has a Fur-like system for gene regulation in response to iron. In addition to the constitutive expression of the 77-kDa HupA protein in the *V. vulnificus fur* mutant, Northern blot analysis and primer extension confirm the regulation of the gene by iron and identify a promoter containing a region homologous to the consensus *E. coli* Fur box.

We previously constructed a *fur* mutant of *V. vulnificus* by in vivo marker exchange (28). This mutant has proved useful so far for studying the acquisition of iron in this pathogen. SDS-PAGE analysis of the *V. vulnificus fur* mutant showed the constitutive expression of at least two outer membrane proteins of approximately 72 and 77 kDa which are normally regulated by iron in wild-type *V. vulnificus*. The 77-kDa protein was overexpressed in sufficient quantities in the *fur* mutant to be separated by SDS-PAGE, isolated, and amino acid sequenced by Edman degradation with a microsequencer. The information from the N-terminal sequence permitted the construction of an oligonucleotide to be used in screening a *V. vulnificus* chromosomal DNA library to clone the gene. The 72-kDa iron-regulated outer membrane protein may also be expressed in sufficient amounts to allow N-terminal amino acid sequencing. Study of this protein could also reveal an additional outer membrane receptor involved in iron uptake.

The oligonucleotide used in this study, although slightly mismatched (8 of 45 nucleotides) for the actual DNA sequence of the gene, was much longer than the original degenerate oligonucleotide (FDEVVV) used in preliminary experiments. This longer oligonucleotide allowed us to clone the *hupA* gene under low-stringency hybridization conditions. Similar to what was reported with cloning the hemoglobin-binding outer membrane protein, HgbA from *Haemophilus ducreyi* (10), we found that clones expressing the full-length *hupA* product grew more slowly and were somewhat unstable. The original clone containing the full-length *hupA* (pCML37) could be maintained only in *E. coli* ABLE K (Stratagene), which reduces the copy number approximately 10-fold, thus decreasing the level of expressed cloned protein product. The *Nco*I-*Bam*HI fragment containing the entire *hupA* gene subcloned in pBluescript could not be transferred into the *V. vulnificus hupA* mutant CML49. Successful complementation of the mutant CML49 could be accomplished only by subcloning the *hupA* gene and its promoter into a pLAFR3 plasmid, which has a much lower copy

TABLE 2. Stimulation of growth of *V. vulnificus* strains by various iron sources and producer strains

Producer strain or iron compound (concn)	Diam of zone of growth (mm) of indicator strain[a]		
	MO6-24 (wild type)	CML49	CML49 (pCML42)
Hemoglobin (10 μM)	15	0	17
Hemin (20 μM)	15	0	16
Transferrin (2.6 mM)	15	12	14
$FeSO_4$ (10 mM)	19	15	17
Vulnibactin (2 mM)	20	18	19
MO6-24	14	13	17
CML49	14	13	16

[a] Cultures were seeded into LB agar containing 75 μg of EDDA per ml, and 5 μl of various iron-containing compounds or overnight growth of a bacterial strain was spotted onto the medium or onto sterile disks placed on the medium. Strains MO6-24 and CML49 were used as producer strains to detect any alteration in the ability to produce siderophores (e.g., vulnibactin) and the ability for the indicator strain to use siderophores as an iron source. The zones of growth around the spots or the disks were measured after 18 to 24 h. Diameter measurements include the size of the disks or spots except in instances of no growth around disks. The measurements represent the average of three experiments.

number than that of pBluescript plasmids. Presumably, *hupA* cloned in a high-copy-number plasmid was lethal in *V. vulnificus*, given the previous difficulty in maintaining *hupA* clones in *E. coli* strains. As shown in Fig. 6, even in a low-copy-number plasmid, the outer membrane protein is expressed in much higher amounts under low-iron conditions than the wild-type or *fur* mutant *V. vulnificus* outer membrane protein and may account for the difficulty in maintaining this clone in a high-copy-number plasmid.

These studies have not necessarily proven that HupA binds hemin directly, but the high degree of homology of HupA with HutA, the heme receptor of *V. cholerae*, suggests that HupA may be the heme receptor of *V. vulnificus*. Mutagenesis of *hupA* in *V. vulnificus* was performed by in vivo marker exchange. Studies on iron utilization using the *hupA V. vulnificus* mutant suggest that HupA is needed for the utilization of hemoglobin and heme. The *hupA* gene cloned on a plasmid was sufficient to complement the defect in heme and hemoglobin utilization, indicating that an internal deletion of *hupA* did not adversely affect any genes adjacent to *hupA*. It is unclear whether HupA could also be a possible hemoglobin receptor. *V. vulnificus* requires a protease for the utilization of hemoglobin (36). A possible mechanism for the utilization of hemoglobin by *V. vulnificus* is that the protease may remove the heme from hemoglobin, thus allowing HupA to bind heme. When the cloned *hupA* gene and its promoter region on a plasmid (pCML42) was transferred into the *V. vulnificus hupA* mutant (CML49), the expression of the protein was regulated by iron, suggesting that the upstream DNA is sufficient for regulation of the gene by Fur and iron. The 77-kDa outer membrane protein as demonstrated by SDS-PAGE (Fig. 6) was expressed to a much higher degree in CML49(pCML42) under low-iron conditions than under high-iron conditions. Complete repression of HupA synthesis was not observed under high-iron conditions. Presumably this is due to multiple copies of *hupA* saturating the limited quantities of the Fur protein expressed in single copy on the chromosome.

When Henderson and Payne (23) used *hutA* DNA sequences from *V. cholerae* to probe chromosomal digests from various *Vibrio* species, they found regions of DNA homology on the chromosome of other *V. cholerae* strains and *Vibrio parahaemolyticus*. They did not, however, find DNA sequences homologous to *hutA* in *V. vulnificus* 324. The nucleotide sequences of *V. vulnificus* MO6-24 *hupA* and *V. cholerae hutA* have 58.3% identity. Therefore, either the stringency conditions of hybridization in the study of Henderson and Payne (23) were not low enough to detect sequences on *V. vulnificus* 324 or the heme receptor DNA sequences on *V. vulnificus* 324 are more disparate than the sequences for *V. cholerae hutA* or *V. vulnificus* MO6-24 *hupA*.

HupA also shows extensive similarity with other TonB-dependent receptors, suggesting that it may also be a TonB-dependent receptor. The proposed TonB box of *V. vulnificus hupA* has substantial similarity with the TonB box from these TonB-dependent outer membrane protein receptors. The *V. vulnificus* TonB is identical to the proposed TonB box of *V. cholerae hutA* except for one amino acid. However, Henderson and Payne (21) found that the *V. cholerae* heme utilization system did not require a functional *E. coli tonB*. On the other hand, the HutA-proposed TonB box only has one of three invariable amino acid residues conserved in the invariable region of known TonB boxes, while HupA has two of the three invariable amino acid residues of the TonB boxes conserved. It would therefore be of interest to test whether *V. vulnificus* requires a functional *E. coli tonB*.

Studies on the virulence of heme utilization mutants of *V. cholerae* showed only a slight reduction of virulence in comparison to that of the wild type or a vibriobactin synthesis mutant (23). Since *V. vulnificus* causes sepsis following oral ingestion and wound infections following seawater exposure, heme acquisition may serve an important role in the pathogenesis of this organism. The hemolysin-cytolysin produced by *V. vulnificus* can lyse erythrocytes and eucaryotic cells, which in turn may free heme-containing compounds to serve as a source of iron during sepsis and wound infections. Future studies involving the analysis of virulence of the *hupA* mutant of *V. vulnificus* compared with the vulnibactin synthesis mutant of *V. vulnificus* should help clarify the role of host iron acquisition in the pathogenesis of this organism.

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