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In pathogenic bacteria, iron acquisition is important for colonization and proliferation in the host under iron-limited conditions. The ability of Vibrio spp. to acquire iron is often critical to their virulence, causing gastroenteritis or excessive watery diarrhea in humans. In the study described here, we cloned the 2,100-bp heme utilization protein gene hupO in Vibrio fluvialis. HupO had high homology to iron-regulated outer membrane receptor proteins in Vibrio sp. and contained motifs that are common to bacterial heme receptors, including a consensus TonB box, a FRAP domain, and an NPRN domain. To characterize the hemin-binding activity of HupO, we purified the recombinant HupO protein (rHupO) from Escherichia coli by using an overexpression system. HupO was found to bind to hemin but not to hemoglobin. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting demonstrated that the 77-kDa outer membrane protein HupO of V. fluvialis was induced under iron-restricted conditions. We constructed a hupO mutant, HP1, to investigate the biochemical function of HupO in V. fluvialis. The hemolytic activity of HP1 was reduced compared to that of wild-type cells and, when exposed to hydrogen peroxide, significantly lower numbers of HP1 survived than was the case in the wild type. These results suggest that HupO is associated with virulence expression in V. fluvialis through stimulation of hemolysin production and resistance to oxidative stress. In experimentally infected mice, the 50% lethal dose value of the wild-type was lower than that of the mutant, HP1.

Iron is an essential element for bacterial growth and the biological processes of most microorganisms, since it is a co-factor of various functional proteins and enzymes and plays an important role in electron transport and redox reactions (9). Most bacteria have evolved iron binding and transport systems to overcome conditions of poor iron availability in the host (55). The best-studied system is that mediated by high-affinity iron chelators and cell surface receptor proteins specific for iron-siderophore complexes. Alternative mechanisms of iron acquisition, such as utilization of heme compounds, are also important for a number of pathogenic bacteria (39, 55). The expression of many iron uptake genes is regulated at the transcriptional level by iron and an iron-binding repressor protein called ferric uptake regulator (Fur). The expression of iron-regulated genes is induced under iron-limited conditions, and many bacterial virulence determinants, including toxins, enzymes, and adhesins, are controlled by environmental iron concentration (29).

Vibrios are gram-negative enteric pathogens that often cause disease in humans (3, 4, 11, 23, 30, 40). Factors involved in the utilization of heme, hemin, and hemoglobin for growth have been reported in species such as Vibrio cholerae, V. vulnificus, and V. anguillarum (21, 22, 33). Under low-iron conditions, V. cholerae and V. parahaemolyticus acquire iron by producing the siderophores vibriobactin (16) and vibrioferrin (56), respectively. Particularly in V. cholerae, iron linked with ferric vibriobactin binds to the vibriobactin receptor, VIuA, a 74-kDa iron-regulated protein, and is then transported into the cell. The transcription of VIuA is regulated by the Fur protein, and the promoter region of the VIuA contains a Fur box. Two proteins, the 77-kDa outer membrane protein (HutA) and the 26-kDa inner membrane protein (HutB), also play a role in siderophore-independent iron acquisition from heme or hemoglobin (22). Other iron-regulated outer membrane proteins, such as the hemin and hemoglobin receptor HutA in V. vulnificus and V. parahaemolyticus, have also been identified (28, 36, 38).

V. fluvialis is a halophilic bacterium that causes gastroenteritis or excessive watery diarrhea in humans and has been associated with enterocolitis in infants (2). It is important as a causative agent of infectious disease, since the clinical symptoms of gastroenteritis caused by V. fluvialis and V. cholerae are very similar. Recently, it has been reported that an enterotoxigenic El Tor-like hemolysin from V. fluvialis bears similarity to the El Tor hemolysin from V. cholerae (25). V. fluvialis produces several other pathogenic factors, such as a factor that prevents the elongation of Chinese hamster ovary (CHO) cells, a cytolysin that induces fluid accumulation in the sucking mouse, a nonhemolytic CHO cell-killing cytotoxin, and proteases (25, 30). Nishibuchi and Seidler showed that production of enterotoxins from V. fluvialis was influenced by the culture medium used to grow this organism (35). Little is known, however, about the utilization of heme compounds by V. fluvialis, although an iron acquisition system mediated by the catecholate siderophore fluvibactin has been reported (57). Expression of iron-regulated proteins in Vibrio spp. has been related to increased virulence in animal models, but the role of heme utilization proteins in bacterial survival under oxidative stress and their effect on the production of pathogenic factors such as hemolysin is unknown.

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We report here the identification of the heme utilization protein HupO, which mediates the acquisition of iron from hemin in \textit{V. fluvialis} and has amino acid sequence homology to bacterial outer membrane heme receptors. We also describe the purification of recombinant HupO protein (rHupO) from \textit{Escherichia coli} by using an overexpression system and its use to investigate hemin-binding activity. We further compare the hemolytic activity and oxidative stress resistance of the wild type and the hupO knockout mutant and examine experimental infections with these strains in mice.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and medium.** The bacterial strains and plasmids used in the present study are described in Table 1. Strains were routinely grown in Luria-Bertani (LB) medium. For preparation of outer membrane proteins in high- and low-iron conditions, LB medium was used with or without the addition of the iron chelator 2,2'-dipyridyl (Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 0.5 \(\mu\)M. Achromobacter \textit{CGA} (45 \(\mu\)g/ml), or chloramphenicol (12.5 \(\mu\)g/ml) was added to the medium as appropriate.

**Cloning procedures and sequencing.** By comparing the hup genes of \textit{V. cholerae} (22) and \textit{V. vulnificus} (28), we designed a set of degenerate primers (sense primer hupO5 \(5'\)-GGGGATAYTGYAAYTTGA-3'; antisense primer hupO4 \(5'\)-GGCATATGTATAACAAATCTTT ACTATC-3'). The amplified DNA fragment was used as a probe for the screening of \textit{V. fluvialis} for the hupO gene. The chromosomal DNA of \textit{V. fluvialis} was fully digested with various restriction enzymes, electrophoresed, and transferred to a nitrocellulose membrane. Southern blot analysis was carried out by using a DIG nonradioactive DNA labeling and detection kit (Boehringer Mannheim GmbH, Mannheim, Germany) under high-stringency conditions. The basis of the Southern blot results analysis, a 5.8-kb HindIII fragment hybridizing with the probe was used for the hupO gene construction. DNA fragments of appropriate length were cut from the agarose gel after electrophoresis and extracted with a gel extraction kit (Nucleogen, Kyonggi, Korea). The insert DNA fragments were ligated into pGEM-4Z cut by NdeI site (underlined) and hupO6 (antisense primer, 5'GGCCAAAGCTTGAATTCGTACTTACGGCG 3'; the HindIII site is underlined). PCR was performed with hupOS and hupO6 primers to amplify the 2.1-kb open reading frame (ORF) of the hupO gene. The PCR product was digested with NdeI and EcoRI, and subcloned into the overexpression vector, pET-22b (+). The recombinant plasmid was named pHUP601 and transformed into \textit{E. coli} BL21(DE3). \textit{E. coli} BL21(DE3) harboring a pHUP601 plasmid was cultivated in 500 ml of LB broth supplemented with 30 \(\mu\)g of ampicillin/ml at 37°C to an optical density at 600 nm of 0.6. HupO expression was induced by the addition of IPTG (isopropyl-beta-thiogalactopyranoside) to a final concentration of 1.0 mM. The cells were harvested and disrupted by sonication. After centrifugation at 10,000 x \(g\) for 30 min at 4°C, the insoluble pellet was suspended in 20 ml Tris-HCl (pH 7.5) containing 6 M urea and incubated at 4°C overnight. The dissolved solution was applied directly to Ni-NTA resin (Qiagen, Valencia, Calif.) equilibrated with 60 mM NiSO4. The proteins were eluted with 1 M imidazole buffer. To recover enzyme activity, proteins were refolded by dialysis.

**Construction of the hupO knockout mutant.** To construct the \textit{V. fluvialis} strain deficient in the production of HupO, a 0.6-kb central fragment of the hupO gene of \textit{V. fluvialis} was amplified by PCR with the primers hupO3 (sense primer, 5'-GGGCGAGCTCATTTACGACCAACAGGATGCAAC-3'; the SalI site is underlined) and hupO4 (antisense primer, 5'-GGGCGCTGAGCTTACGGCAAAAGACT-3'). The amplified fragment was digested with SalI and cloned into pET-22b (+). The resulting plasmid, pHUP600, encoded the 5'-3'-truncated hupO gene. This recombinant plasmid was introduced into the conjugal donor \textit{E. coli} SM10. Conjugation was carried out between the recipient \textit{V. fluvialis} and the donor \textit{E. coli} strain containing pHUP600. A conjugant carrying a single-crossover mutation of hupO was obtained by selection on thiosulfate-citrate-bile salts agar containing chloramphenicol and was confirmed to have an insertion into the hupO gene by Southern blotting with the same probe. The following procedures were added in sequence and gently mixed with the cells: 0.5 ml of 0.2 M Tris-HCl (pH 8.0), 1 M sucrose, 0.05 ml of 10 mM EDTA (pH 8.0), 0.05 ml of l-lysine (2 mg/ml), and 1.6 ml of H2O. The cells were then kept for 5 to 10 min at room temperature to convert most cells to spheroplasts. After this period, 2.5 ml of extraction buffer (50 mM Tris-HCl [pH 8.0], 2% [vol/vol] Triton X-100, 10 mM MgCl2) was added. The cleared solution was mixed thoroughly and kept for 5 min at room temperature until it was no longer viscous. The solution was then centrifuged at 40,000 \(\times\) \(g\) for 1 h at 4°C. The pellet was resuspended in 1.5 ml of water, transferred to an Eppendorf tube, and centrifuged for 15 min. The outer membrane was washed three times with 1.5 ml of water and then used for gel electrophoresis.

**Hemin- and hemoglobin-binding assay with isolated outer membrane proteins**

**TABLE 1. Strains and plasmids used in this study**
and rHupO. A hemin- and hemoglobin-agarose-binding assay was conducted essentially as described by Lee (27). Briefly, 200 μl of hemin- or hemoglobin-agarose (Sigma Chemical Co.) was washed with 600 μl of Tris buffer (25 mM [pH 7.4]) containing 100 mM NaCl. Washing was performed three times by suspension of the agarose in 1 ml of buffer, followed by centrifugation at 10,000 × g for 5 min. The isolated outer membrane proteins from V. fluvialis were incubated with the hemin- or hemoglobin-agarose for 5 h at 37°C with gentle mixing. The sample was centrifuged at 10,000 × g for 5 min, and the supernatant was removed. The agarose was washed three times as described above, and bound proteins were eluted by incubation for 2 min with 100 μl of 2 M guanidine-HCl, which was separated from the agarose beads by centrifugation at 10,000 × g for 5 min. A negative control, omitting the hemin- or hemoglobin-agarose, was incubated and treated in an identical method. Proteins in the 2 M guanidine-HCl eluants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

The hemin-binding activity of purified HupO was measured by a standard liquid hemin-binding assay. For the hemin-binding assay, samples were incubated at 37°C for 1 h and then centrifuged, the supernatant transferred to the wells of microplates, and the absorbance of the unbound hemin in the supernatant measured at 405 nm in a microplate reader (Bio-Rad Laboratories, Richmond, Calif.). Compounds diluted in phosphate-buffered saline (PBS) were incubated under the same conditions and served as appropriate controls. The binding of all compounds was determined by the decrease of absorbance of the hemin- or hemoglobin-agarose for 5 h at 37°C with gentle mixing. Samples were collected at 10, 20, 30, 40, 50, and 60 min after the addition of H2O2. Appropriate bacterial dilutions were plated onto LB agar plates. The percentage survival was calculated by dividing the number of CFU at different time points by the initial number of CFU at time zero. Experiments were performed four to five times with duplicate samples.

Virulence assays. We performed oral challenge of infant mice to measure the degree of stimulation of gastroenteritis and mortality as shown in studies of enterotoxigenic V. cholerae (50). To investigate the virulence of the wild-type and Hp1 in the mouse, 7- to 10-day-old female CD-1 mice were used. Each bacterial strain was resuspended in PBS and injected orally at different concentrations. Ten mice were used per bacterial dose. Mortality was checked up to 48 h. The degree of virulence was expressed as the 50% lethal dose (LD50). The effect of hemin as a potential adjuvant on the virulence of isolates was tested by a modification of the method of Frouz et al. (13). Groups of mice pretreated and nontreated with hemin were compared. At 2 h before bacterial infection, mice were inoculated orally with 0.25 μg of hemin per g of body weight. Groups of four mice were injected orally with 30 μl of a 10-fold dilution series of bacterial suspensions (101 to 105 CFU/ml strain) in saline. The LD50 values were calculated by the method of Reed and Muench (42). Differences within the data were evaluated for significance by using Student t test. P values of <0.01 were considered statistically significant.

Nucleotide sequence accession number. The nucleotide sequence of the hupO ORF has been deposited in the GenBank database under accession number AY560602.

RESULTS

Cloning of V. fluvialis hupO gene and comparison with Hup-related amino acid sequences in Vibrio spp. In order to detect the presence of HupO in V. fluvialis, we performed Southern blot analysis. To design the probe, we used the sequences of the iron-regulated genes hutA and huhA from V. cholerae and V. vulnificus. Southern blot hybridization analysis of V. fluvialis chromosomal DNA showed that a 5.8-kb HindIII-digested fragment hybridized with the probe (data not shown). This 5.8-kb HindIII fragment was successfully cloned and plasmid DNA named pHUP580 was isolated. The complete nucleotide sequence of the insert in pHUP580 was determined. Translation of the nucleotide sequence revealed an ORF of 2,100 bp, encoding a polypeptide of 700 amino acid residues, with a calculated molecular mass of 77,592 Da. The proposed ATG trans-
The translation start codon is preceded by a probable ribosome-binding site, AAGG. An inverted repeat, which could function as a transcription termination signal, was found 48 bp downstream from the stop codon.

Alignment of the deduced hup-related amino acid sequence with four hup homologues from *V. anguillarum* (33), *V. vulnificus* (28), *V. parahaemolyticus* (38), and *V. cholerae* (22) showed 60, 56, 51, and 51% identities, respectively. The nucleotide sequence homologies were 65, 62, 62, and 60%, respectively.

Expression and purification of rHupO from *E. coli*. To characterize the hemin-binding activity of HupO, the rHupO protein was purified by a His tag method. Purified rHupO showed a single band on SDS-PAGE and had an estimated molecular mass of ca. 77 kDa (Fig. 2). About 2 mg of pure rHupO was recovered from 75 mg of total cell lysate.

![FIG. 2. SDS-PAGE of purified rHupO. Lanes are as follows: M, molecular size markers; lane 1, crude extract of cells with pET22(+)1; lane 2, cells cultured for 4 h after induction of transcription of rHupO; lane 3, inclusion body isolation; lane 4, His tag column elution fraction.](image)

![FIG. 3. SDS-PAGE profiles and Western blotting with anti-rabbit serum to rHupO. (A) SDS-PAGE analysis of outer membrane proteins of *V. fluvialis* and mutant strain HP1. Lanes: M, molecular size markers; 1, wild-type *V. fluvialis* grown in high-iron medium; 2, wild-type *V. fluvialis* grown in low-iron medium; 3, mutant strain HP1 grown in high-iron medium; 4, mutant strain HP1 grown in low-iron medium. (B) Western blot analysis. Lanes: 1, wild-type *V. fluvialis* grown in high-iron medium; 2, wild-type *V. fluvialis* grown in low-iron medium; 3, mutant strain HP1 grown in high-iron medium; 4, mutant strain HP1 grown in low-iron medium. The asterisk indicates the position of the 77-kDa HupO protein.](image)
Construction of a mutant of *V. fluvialis* with an internal deletion of *hupO*. We used a standard suicide vector method to insertionally inactivate the *hupO* gene of the wild-type *V. fluvialis* strain. The insertional disruption of *hupO* in the mutant was confirmed by Southern blot analysis (data not shown). When wild-type *V. fluvialis* genomic DNA digested with EcoRI was hybridized with the internal coding sequence probe that cloned the SalI and SacI fragment of the *hupO* gene, a 1.7-kb band was observed. Strain HP1 showed 1.0- and 1.4-kb hybridizing bands (data not shown). This pattern of hybridization confirms that the *hupO* gene of HP1 was disrupted by the insertion of pHUP600 plasmid DNA. Also, PCR analysis was performed to support the results of Southern hybridization. We additionally confirmed the inactivation of *hupO* by comparing the outer membrane proteins of wild-type *V. fluvialis* and HP1 after growth in low- and high-iron media.

**Analysis of the iron regulator outer membrane protein of *V. fluvialis* and mutant HP1.** The outer membrane protein profiles of the wild-type strain and HP1 grown to late-log phase in LB with or without 0.2 mM 2,2'-dipyridyl were compared by SDS-PAGE and subsequent immunoblot analysis. In the wild-type strain, two proteins with apparent molecular sizes of 72 and 77 kDa appeared after growth under low-iron conditions. The outer membrane protein profiles of the mutant HP1 showed a loss of expression of the 77-kDa protein under low-iron conditions (Fig. 3A). The 77-kDa outer membrane protein induced in *V. fluvialis* under iron-limited condition was verified as HupO by Western blotting analysis (Fig. 3B).

**Characterization of hemin-binding activity of HupO.** The hemin- and hemoglobin-binding characteristics of the isolated outer membrane protein from *V. fluvialis* were examined by using affinity chromatography with hemin- and hemoglobin-agarose. HupO was identified from a mixture of the outer membrane proteins of *V. fluvialis* by using Western blot analysis of proteins that bound to hemin-agarose. Proteins eluted from the hemin-agarose with 2 M guanidine-HCl were separated by SDS-PAGE and then subjected to Western blot analysis with HupO-specific antiserum. This analysis revealed a single band of 77 kDa (Fig. 4A), which was not seen in the negative control. When hemoglobin-agarose was used for binding, however, no band was detected.

In the growth stimulation assay, although both strains grew equally well with exogenously supplied hemoglobin, significant growth stimulation differences were observed when hemin was used as the sole iron source (data not shown). The wild-type strain formed colonies around the disk containing hemin but HP1 failed to grow. This result supports the hypothesis that HupO binds specifically to hemin. Figure 4B shows the binding of hemin to rHupO in a concentration-dependent fashion. The binding approached saturation with increasing concentrations of rHupO. Scatchard analysis of hemin binding to rHupO yielded a linear plot with a binding affinity ($K_d$) of $4.6 \times 10^{-5}$ M.

**Comparison of hemolytic activity.** Hemolysin activity was measured on the culture supernatants from wild-type and HP1 strains by the plate hemolysin assay (Fig. 5). Hemolytic activity was almost undetectable in HP1 strains. To confirm this finding, hemolytic activity was measured by using the hemolysin

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**FIG. 4. Hemin-binding activity of outer membrane proteins and purified rHupO.** (A) Western blot analysis, with HupO-specific antiserum, of *V. fluvialis* outer membrane proteins eluted from hemin-agarose with 2 M guanidine-HCl. Lanes: 1, negative control; 2, hemin-agarose eluant. (B) Saturation of hemin binding and Scatchard plot analysis (inset) of rHupO. rHupO was resuspended in PBS and incubated for 1 h at 37°C with hemin. Binding was determined by the decrease of absorbance of the supernatant at 405 nm and recorded as the percent input of hemin. Three independent experiments were performed in triplicate. The data are means ± the standard deviation.

**FIG. 5. Hemolytic activity of wild-type (A) and mutant strain HP1 (B) on blood agar.** Cells were removed by centrifugation, and the supernatant was sprinkled onto a punched blood agar plate.
assay as described in Materials and Methods. The hemolytic activities of the wild-type strain and HP1 were 178 and 70 HU per mg of total supernatant protein, respectively. The wild-type strain showed much more hemolytic activity under iron-depleted conditions (data not shown).

**Sensitivity to oxidative stress.** To investigate the role of HupO in the resistance of *V. fluvialis* to oxidative stress, we examined the effect of hydrogen peroxide on cell viability. The HP1 mutant was hypersensitive to hydrogen peroxide exposure, compared to the wild-type strain. After a 20-min incubation in 5 mM H$_2$O$_2$, the viable cell numbers of the wild-type strain were ~10-fold higher than that of the mutant. After 30 min of H$_2$O$_2$ exposure, the wild-type strain was reduced by 10$^2$-fold and still persisted after 60 min of exposure, but little HP1 was detected (Fig. 6). This result suggests that the HupO protein of *V. fluvialis* is involved in resistance to conditions of oxidative stress.

**Iron availability and virulence.** The role that HupO plays in the virulence of *V. fluvialis* was tested by experimental infections in mice. The degrees of virulence of wild-type and mutant HP1 strains were compared. The LD$_{50}$ of wild-type strain was reduced compared to that of the mutant HP1. When mice were previously overloaded with hemin, the LD$_{50}$ for untreated mice of wild-type strain decreased significantly. The degree of virulence of the mutant HP1, however, was not affected by previous inoculation of mice with hemin (Table 2). Thus, mutation of the heme utilization protein greatly affects the virulence properties of *V. fluvialis* under low-iron conditions.

### DISCUSSION

Most pathogens have evolved diverse systems for acquiring iron in the iron-limited environment of the host (55). Among the mechanisms of iron acquisition, the ability to utilize heme compounds appears to be particularly important in pathogenic bacteria (39, 55). The expression of genes involved in iron acquisition is regulated by iron or hemin concentration. HupO of *V. fluvialis* has high homology to iron-regulated outer membrane receptor proteins in *Vibrio* spp., which have been reported to interact with different substrates. *V. vulnificus* HupA and *V. anguillarum* HuvA can utilize hemin and hemoglobin, unlike *V. cholerae* HutA and *V. parahaemolyticus* HutA, which can take up iron only from hemin (14). In the present, we found that HupO of *V. fluvialis* binds specifically to hemin.

A well-known mechanism by which bacteria acquire heme involves direct binding of heme or heme proteins to specific outer membrane receptors (14). After this event, heme is removed from the bacterial receptor and iron is transported into the cell. The transport of iron across the outer membrane in most gram-negative bacteria requires energy. It is thought that the energized conformation of TonB, which is anchored in the inner membrane induced by proton motive force causes a conformational change in transport proteins. Outer membrane receptors are provided with the required energy by the TonB system and share amino acid homology in several regions termed “TonB boxes.” In most gram-negative pathogenic bacteria that have been examined, a functional TonB protein is required for heme and hemoglobin utilization (5). In *V. fluvialis*, predicted TonB box amino acid sequences, which may be involved in direct interaction with the TonB protein, are detected near the N terminus of Hut (DEVVVS) (Fig. 1A). Also, the amino acid sequence of the carboxyl-terminal region showed significant homology with the HuvA of *V. anguillarum*.

Iron-regulated gene expression in gram-negative bacteria is generally under the control of the Fur protein, which represses the transcription of iron-regulated promoters in response to an increasing intracellular Fe$^{2+}$ concentration. Fur globally regulates a variety of iron-dependent cellular processes, such as the acid-shock response (12) and the oxidative-stress response (49), and also regulates the genes involved in the biosynthesis and uptake of several siderophores (17). In addition, Fur modulates, at least in part, the expression of alternative sigma factor and activator genes (37) and other virulence-associated genes (6, 15, 17, 52). By nucleotide sequence analysis, a putative Fur box was found in the upstream region of the *hupO* gene, and we showed that this protein is more highly expressed under low-iron conditions than under high-iron conditions. As shown in Fig. 3, two proteins with apparent molecular sizes of 77 kDa (HupO) and 72 kDa appeared in *V. fluvialis* after growth under low-iron conditions. This result is consistent with the finding that *V. vulnificus* expressed at least two iron-regulated outer membrane proteins of ca. 77 and 72 kDa (7). These 77- and 72-kDa proteins have been observed to correspond to the heme uptake receptor (HupA) and the vulnibactin receptor (VuuA) (28), respectively.

It has been reported that a variety of virulent bacteria dem-

### TABLE 2. Virulence for mice of *V. fluvialis* strain and HP1 strain

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<tr>
<th>Strain</th>
<th>Virulence* (mean LD$_{50}$ [CFU/mouse]) in:</th>
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<tbody>
<tr>
<td></td>
<td>Mice not treated with hemin</td>
<td>Mice pretreated with hemin</td>
</tr>
<tr>
<td>Wild type</td>
<td>$&gt;2.1 \times 10^{10}$</td>
<td>$&gt;1.1 \times 10^7$</td>
</tr>
<tr>
<td>HP1</td>
<td>$&gt;9.3 \times 10^{10}$</td>
<td>$&gt;5.8 \times 10^{10}$</td>
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* Mean LD$_{50}$ values are expressed as CFU per mouse ($P < 0.01$).
onstrate increased hemolysin production under iron-limited conditions. In *Vibrio* spp., hemolysin production by *V. cholerae* and *V. parahaemolyticus* were found to be enhanced under iron-limited conditions, increasing cytotoxicity to intestinal epithelial cells and concurrent lysis of erythrocytes (8, 44). In the present study, we examined whether expression of HupO affects hemolytic activity by using a hupO gene knockout mutant. Hemolytic activity of the HP1 mutant was significantly reduced (Fig. 5). HupO was induced under iron-limited conditions, when the hemolytic activity of the wild-type strain was enhanced. The results suggest that the enhanced hemolytic activity liberates hemin from intracellular hemoglobin and makes it available for use by organisms for growth, so hemolytic activity plays a beneficial role in bacterial iron acquisition in the host. This would be consistent with a similar role described for the hemin-binding protein HBP35 in *Porphyromonas gingivalis*. Since HBP35 is essential for heme uptake, a HBP35-deficient mutant showed severe reductions in virulence properties such as hemolysis and hemagglutination (43).

According to Touati (51), there is an intimate relationship between oxidative stress and iron metabolism. Oxidative damage to DNA, RNA, proteins, and the cell membrane occurs when the cellular concentration of reactive oxygen species exceeds the capacity of the cell to eliminate them. As a defense, cells have evolved inducible responses to protect themselves against oxidative stress. Therefore, resistance to oxidative stress has been considered an important virulence factor (10, 34, 47). We tested the survival of the wild-type and HP1 strains under oxidative stress and found that the resistance to H$_2$O$_2$ of the mutant HP1 was significantly reduced compared to the wild-type strain. To investigate whether *V. fluvialis* HupO is involved with catalase expression and affects hydrogen peroxide sensitivity, we conducted native gel electrophoresis and stained gels by the method of Wayne and Diaz to visualize bands of catalase activity (54). There was no difference in catalase activity between the wild type and HP1 (data not shown). This result suggested that the reduced resistance to oxidative stress in HP1 is not related to catalase expression. Further studies are needed to determine how HupO is involved in sensitivity to oxidative stress.

Our results showed differences in hemolytic activity and survival under oxidative stress between the HupO-deficient mutant and wild-type strains. It is known that the enterotoxigenic hemolysin of *Vibrio* spp. plays a role in the disease process in animals (25). We therefore expected to see a difference in virulence in mice between the two strains. The results of the present study showed an increase in the LD$_{50}$ value of the HP1 mutant. We also compared the colonization of the mouse intestine by the wild-type strain and HP1. The mutant HP1 showed a 10-fold reduction in numbers in the intestine compared to the wild-type strain (data not shown). The heme uptake system may provide a clear advantage in the colonization of host tissues under low iron conditions. The result of the present study is consistent with the link found between heme uptake and virulence in several bacteria such as *V. cholerae*, "Pasteurella piscicida," *Haemophilus ducreyi*, *Neisseria meningitidis*, and *Streptococcus pneumoniae* (1, 22, 32, 46, 48).

In summary, *V. fluvialis* HupO was found to be induced under iron-limited conditions and to utilize hemin as an iron source. The HupO protein was involved with hemolytic activity and oxidative stress sensitivity and was associated with virulence by measuring of LD$_{50}$ of *V. fluvialis* in mice. Future studies of the virulence of the hupO-deficient mutant of *V. fluvialis* should help to clarify the role of host iron acquisition in the pathogenesis of this organism.

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