Characterization of a *Vibrio vulnificus* LysR homologue, HupR, which regulates expression of the haem uptake outer membrane protein, HupA

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(Received April 2, 2001; accepted in revised form September 18, 2001)

In *Vibrio vulnificus*, the ability to acquire iron from the host has been shown to correlate with virulence. Here, we show that the DNA upstream of *hupA* (haem uptake receptor) in *V. vulnificus* encodes a protein in the inverse orientation to *hupA* (named *hupR*). HupR shares homology with the LysR family of positive transcriptional activators. A *hupA-lacZ* fusion contained on a plasmid was transformed into Fur−, Fur+, and HupR− strains of *V. vulnificus*. The β-galactosidase assays and Northern blot analysis showed that transcription of *hupA* is negatively regulated by iron and the Fur repressor in *V. vulnificus*. Under low-iron conditions with added haemin, the expression of *hupA* in the *hupR* mutant was significantly lower than in the wild-type. This diminished response to haem was detected by both Northern blot and *hupA-lacZ* fusion analysis. The haem response of *hupA* in the *hupR* mutant was restored to wild-type levels when complemented with *hupR* in trans. These studies suggest that HupR may act as a positive regulator of *hupA* transcription under low-iron conditions in the presence of haemin.

**Key words:** *Vibrio vulnificus*, haem receptor, LysR, Fur, iron, HupR.

Introduction

*Vibrio vulnificus* is a halophilic, marine pathogen that has been associated with septicaemia and serious wound infections in patients who have iron overload, haemochromatosis, cirrhosis or alcoholism [1–5]. Septicaemia is often acquired by eating shellfish and mortality rates of patients with septicaemia often exceed 50% [6]. Wound infections are associated with exposure of wounds to seawater [7].

The ability to make use of available host iron is an important determinant for pathogenicity in many bacteria. Much of the extracellular iron within eukaryotes is sequestered by iron-binding proteins such as transferrin and lactoferrin, while intracellular iron is predominantly bound to haem [8]. To overcome this iron sequestration,
bacterial pathogens have evolved a number of mechanisms to extract iron from host iron-binding compounds. Many pathogens produce siderophores, low molecular weight iron-chelating molecules, that can remove iron from transferrin and lactoferrin [9, 10]. Numerous pathogens also possess mechanisms for obtaining iron from haem and haem-containing proteins [11]. The ability to acquire iron appears to be particularly important in the pathogenesis of V. vulnificus infections and the virulence of V. vulnificus has been directly correlated with iron availability. The injection of iron into mice has been reported to lower the 50% lethal dose of a virulent strain of V. vulnificus [12]. V. vulnificus is also able to use host iron such as transferrin, haemoglobin, haem and haemoglobin/haptoglobin complex [13], and the lethality of intraperitoneal inocula of V. vulnificus is increased by concurrent injections of haemoglobin and haematin [13].

The expression of many iron-uptake genes is controlled at the transcriptional level by iron and an iron-binding repressor protein called Fur (ferric uptake regulation) [14]. Exceptions to the regulation by iron occurs in pathogenic Haemophilus species where the synthesis of haemoglobin- and transferrin-binding proteins is haem repressible and unaffected by the iron concentration [15–17]. Haem transport in Porphyromonas gingivalis is also thought to be regulated by haem [18, 19]. In Corynebacterium diphtheriae the hmuO gene encodes a haem oxygenase that is involved in the utilization of haem and haemoglobin as iron sources [20].

Transcription of the hmuO gene in C. diphtheria is controlled under a dual regulatory mechanism in which the diphtheria toxin repressor protein (DtxR) and iron repress expression, while either haem or haemoglobin is needed to activate transcription [21].

We previously cloned and characterized the haem receptor of V. vulnificus (hupA) [22]. We have found that the DNA upstream of hupA encodes a protein in the inverse orientation to hupA (named hupR). HupR shares homology with the LysR family of positive transcriptional activators. LysR positive transcriptional activators are often coinducer-responsive proteins that are divergently transcribed from a promoter that is very close to the promoter of the regulated target gene. In this study we analysed the mechanism of regulation of transcription of hupA by HupR and haemin.

Results

Northern blot analysis of the open reading frame upstream of HupA

On sequence analysis we found an open reading frame upstream of hupA in the inverse orientation to hupA (named hupR). A restriction map demonstrating this region and subclones used in the study is described in Fig. 1. Northern blot analysis of RNA was performed to determine whether an RNA transcript was associated with the upstream open reading frame and to
**Figure 2.** Northern blot analysis of RNA prepared from *V. vulnificus* MO6–24 after growth in high-iron medium (lane 1) and low-iron medium (lane 2), from *V. vulnificus* 80363 after growth in high-iron medium (lane 3) and low-iron medium (lane 4) and *V. vulnificus* CML17 after growth in high-iron medium (lane 5) and low-iron medium (lane 6). Lanes 1–6 contain 10 μg of RNA each. Lanes 7–9 contain increasing concentrations of RNA as a control for exposure within the linear range. Lane 7 contains 5 μg RNA, MO6–24 low-iron medium; lane 8, 10 μg RNA, MO6–24 low-iron medium; lane 9, 15 μg RNA, MO6–24 low-iron medium. The blot was probed with a 595 bp BglII-EcoRI fragment internal to *hupR*. The positions of single-stranded RNA molecular weight markers (in kb) are indicated on the left.

Vibrio vulnificus HupR

Determine the size of the transcript, if present. Northern blot analysis of RNA prepared from MO6–24 following growth in low- and high-iron media was performed also to determine whether or not transcription was regulated by iron (Fig. 2). The blot was probed with a DNA fragment internal to the open reading frame. A single band less than 1.3 kb in size was seen in RNA prepared from MO6–24 grown under both high- and low-iron conditions (Fig. 2). The gene was designated *hupR*. The intensity of the bands were compared densitometrically and areas were expressed as pixels. The intensity of the band was increased under low-iron conditions almost two-fold (1.6 K pixels, high-iron; 3.0 K pixels, low-iron) suggesting negative regulation by iron. Northern blot analysis was also performed on RNA prepared from the *fur* mutant CML17 and the parent strain of CML17, 80363, following growth in low- and high-iron media to determine of transcription was regulated by Fur (Fig. 2, 80363, lanes 3 and 4; CML17 lanes 5 and 6). The band seen in the *fur* mutant did not differ greatly in intensity between low- and high-iron conditions (3.8 and 3.6 K pixels, respectively), suggesting that *hupR* is regulated by iron via the Fur repressor. Strain 80363 showed only a modest increase in intensity in low-iron conditions vs high-iron conditions (2.2 and 1.4 K, respectively) suggesting there may be strain to strain variation of the regulation of *hupR* by iron.

RNA from each sample (10 μg) was loaded in lanes 1–6. Lanes 7–9 contain increasing amounts of RNA from MO6–24 grown under low-iron conditions (5, 10, 15 μg) to control for exposure of the Northern blot within the linear range. A plot of the expression of *hupR* RNA in MO6–24 grown in low-iron media (1.7, 2.6, 3.4 μg) against the amount loaded (5, 10, 15 μg) showed a calculated r value of 0.999, indicating that exposures of the Northern blots were within the linear range.

**Localization of the start site of *V. vulnificus* hupR transcription**

Primer extension analysis of RNA from *V. vulnificus* MO6–24 grown under low-iron conditions was done by using a synthetic oligonucleotide complementary to the DNA sequence near the ribosomal binding site (Fig. 3, bases 289–269). A primer extension product corresponding to basedetermine the size of the transcript, if present. Northern blot analysis of RNA prepared from MO6–24 following growth in low- and high-iron media was performed also to determine whether or not transcription was regulated by iron (Fig. 2). The blot was probed with a DNA fragment internal to the open reading frame. A single band less than 1.3 kb in size was seen in RNA prepared from MO6–24 grown under both high- and low-iron conditions (Fig. 2). The gene was designated *hupR*. The intensity of the bands were compared densitometrically and areas were expressed as pixels. The intensity of the band was increased under low-iron conditions almost two-fold (1.6 K pixels, high-iron; 3.0 K pixels, low-iron) suggesting negative regulation by iron. Northern blot analysis was also performed on RNA prepared from the *fur* mutant CML17 and the parent strain of CML17, 80363, following growth in low- and high-iron media to determine of transcription was regulated by Fur (Fig. 2, 80363, lanes 3 and 4; CML17 lanes 5 and 6). The band seen in the *fur* mutant did not differ greatly in intensity between low- and high-iron conditions (3.8 and 3.6 K pixels, respectively), suggesting that *hupR* is regulated by iron via the Fur repressor. Strain 80363 showed only a modest increase in intensity in low-iron conditions vs high-iron conditions (2.2 and 1.4 K, respectively) suggesting there may be strain to strain variation of the regulation of *hupR* by iron.

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**DNA sequence of *hupR* and deduced protein sequence of HupR**

The nucleotide sequence of *hupR* 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 885 bp open reading frame begins 82 bp downstream from the putative start site of transcription. A Shine–Dalgarno sequence is indicated just upstream of the initiating GTG start codon. HupR is 295 amino acids in length and has a predicted pl of 6.46 and a predicted molecular weight of 32 825.

A possible transcriptional terminator is identified 257 bp downstream of the stop codon consisting of a six bp, G-C rich, perfect inverted repeat separated by seven bases and followed with a run of six T residues. The proposed start size, 82 bp upstream of the 885 bp open reading frame, and the transcriptional terminator 257 bp downstream of the stop codon, predict an RNA transcript of approximately 1.22 kb, which is consistent with the 1.3 kb *hupR* transcript seen on Northern blot (Fig. 2).
Figure 3. Partial nucleotide sequence of *V. vulnificus* hupR, including the detail of the promoters of hupR and hupA. The promoter of hupR (−35, −10), the approximate start site of transcription (*). The ribosomal-binding site (RBS) and the deduced amino acid sequence are indicated on the upper strand, while the corresponding details of hupA are noted on the bottom strand. The region homologous to the *E. coli* Fur-binding site (FUR BOX) is enclosed within a box. Arrows indicate the direction of transcription. A perfect dyad symmetric element is indicated by heavy horizontal arrows next to the −35 binding site of the hupA promoter. An interrupted inverted repeat is indicated by light horizontal arrows.

Homology of *V. vulnificus* HupR to the LysR family of positive transcriptional activators

Comparison of the amino acid sequence of HupR to other proteins using the BLAST algorithm demonstrated significant homology between HupR and the LysR family of positive transcriptional activator proteins in bacteria, especially in the N-terminus [23]. Homology was seen with TrpI of *Pseudomonas aeruginosa* (GenBank accession #P76594; 22% identity), IlvY of *Escherichia coli* (PO5827; 21% identity), AmpR of *Enterobacter cloacae* (PO5051; 20% identity), IrgB of *Vibrio cholerae* (P25543; 22% identity) and HlyT of *V. cholerae* (P52692; 20% identity). The homology near the amino terminus of HupR with several members of the LysR family is shown in Fig. 4. Using the algorithm of Dodd and Egan, a helix-turn-helix motif was seen in the same region of HupR as in the other members of the LysR family (Fig. 4) [23, 24].

Identification of a putative HupR-binding promoter sequence

Most characterized LysR type proteins bind to their regulated promoters at a recognition site which consists of an approximately 15 bp, partially dyadic sequence centered near −65 with respect to the target gene, which has the conserved T-N₁₁-A motif. Binding also often involves symmetrical guanine bases in the two dyad arms. LysR type transcription activators also interact with an activation site containing a sequence dissimilar to the recognition site. This recognition site also shows dyad symmetry and is located near the −35 RNA polymerase binding site for the target gene [23].
A 13 bp interrupted inverted repeat sequence (3’GTAC-N5GTACA5’; Fig. 3, light horizontal arrows) centered at the –65 region (with respect to hupA) has been identified in the sequence and has a conserved T-N11-A motif. This sequence may be a potential recognition sequence for regulation of hupA. A perfect inverted repeat is also noted near the proposed –35 box of hupA, and may be a good candidate for a LysR-type activation site (Fig. 3; heavy horizontal arrows).

Construction of a mutant of V. vulnificus with an internal deletion of hupR (strain CML55)

A hupR deletion mutant was constructed by in vivo marker exchange. The hupR deletion mutant CML55 was tested for its ability to use haemin, haemoglobin transferrin, FeSO₄, and vulnibactin. No differences were seen in the ability to use the various iron sources between CML55 and wild-type MO6–24 as measured zones of growth around disks containing 1.25 μg of haemoglobin, 10 μM, haemin (20 μM), transferrin (2.6 mM), FeSO₄ (10 mM) and vulnibactin (2 mM) in LB chelated with EDDA (75 μg/ml) seeded with the V. vulnificus strains. Zone sizes were as follows: haemoglobin, 17 ± 1 MO6–24, 16 ± 1 CML55; haemin, 17 ± 1 MO6–24, 16 ± 1 CML55; transferrin, 14 ± 2 MO6–24, 13 ± 2 CML55; FeSO₄, 18 ± 1 MO6–24, 17 ± 1 CML55; vulnibactin, 19 ± 1 MO6–24, 16 ± 2 CML55.

Northern blot analysis of hupA transcript in wild-type MO6–24 compared to hupR mutant CML55

Northern blot analysis was performed with RNA prepared from V. vulnificus wild-type MO6–24 and CML55, and CML55 containing hupA in trans (pCML55) grown in low-iron (LB plus 0.1 mM 2,2’-dipyridyl), high-iron (LB media alone) and low-iron with added haemin (LB plus 0.1 mM, 2,2’-dipyridyl and 40 μM haem). RNA from each sample (10 μg) was loaded in each lane. The blot was probed with the BglII-HindIII fragment contained in the V. vulnificus hupA gene [Fig. 5(a)]. To control for indirect effects of haemin as an iron source and as an internal control for quantitation, a second Northern blot using the same RNAs in the first blot were probed with the BglII/EcoRV fragment containing the V. vulnificus vuuA gene (vulnibactin receptor) [25]. RNA from each sample (10 μg) was loaded in each lane except the last four lanes, 10–13, in which increasing amounts of RNA (1.25, 5, 10, 15 μg) was loaded to control for exposure of the blots within the linear range.

No transcripts were seen under high-iron conditions for either Northern blot probed with hupA or vuuA. Transcripts of approximately 2400 bases were observed under low-iron and low-iron plus haemin conditions in both wild-type MO6–24 and the hupR mutant CML55 in the blot probed with hupA [Fig. 5(a)]. The intensity of the bands were examined densitometrically. Expression of hupA under the low-iron condition alone did not differ significantly, for the wild-type vs the hupR mutant and hupR mutant containing hupR in trans (12.0, 12.0, 13.0 K pixels by densitometry, respectively). Over a 40% reduction of hupA expression was observed in the hupR mutant under low-iron plus haemin conditions (5.2 K pixels) when compared to wild-type MO6–24 under low-iron plus haemin conditions (9.1 K pixels). Expression of hupA returned to wild-type levels in the hupR mutant containing hupR in trans (10.8 K pixels).

Expression of vuuA under low iron conditions did not differ considerably, for the wild-type vs

Figure 4. Homology between the amino terminus of HupR and several members of the LysR family of positive transcriptional activators. Amino acids are identified in single letter code and identical residues are in bold text. The conserved helix-turn-helix domain of these proteins is indicated above the sequences.
Figure 5. (a) Northern blot analysis of RNA from MO6–24, CML55 and CML55 (pCML55) probed with a DBA fragment internal to hupA. Lane 1, MO6–24 grown in high-iron medium; lane 2, CML55 high-iron medium; lane 3, CML55 (pCML55) high-iron medium; lane 4, MO6–24 low-iron medium; lane 5, CML55 low-iron medium; lane 6, CML55 (pCML55) low-iron medium; lane 7, MO6–24 low-iron medium with 40 μM haemin; lane 8, CML55 low-iron medium with 40 μM haemin; lane 9, CML55 (pCML55) low-iron medium with 40 μM haemin. (b) Northern blot analysis of RNA from MO6–24, CML55 and CML55 (pCML55) probed with a DNA fragment internal to vuuA. Lane 1, MO6–24 grown in high-iron medium; lane 2, CML55 high-iron medium; lane 3, CML55 (pCML55) high-iron medium; lane 4, MO6–24 low-iron medium; lane 5, CML55 low-iron medium; lane 6, CML55 (pCML55) low-iron medium; lane 7, MO6–24 low-iron medium with 40 μM haemin; lane 8, CML55 low-iron medium with 40 μM haemin; lane 9, CML55 (pCML55) low-iron medium with 40 μM haemin; lane 10, 1.25 μg RNA, MO6–24 low-iron medium; lane 11, 5 μg RNA, MO6–24 low-iron medium; lane 12, 10 μg RNA, MO6–24 low-iron medium; lane 13, 15 μg RNA, MO6–24 low-iron medium. The positions of single-stranded RNA molecular weight markers (in kb) are indicated on the left.

the hupR mutant and the hupR mutant in trans (16.6, 19.2, 11.9 K pixels by densitometry, respectively). The expression of vuuA was reduced considerably relative to hupA transcript, when haemin was added as an iron source to the low-iron media. The values did not differ significantly for the wild-type vs the hupR mutant and hupR mutant containing hupR in trans (2.6, 2.1, 3.5 K respectively). A plot of the expression of vuuA RNA in wild-type grown in low-iron media (2.3, 11.6, 18.5, 21.5 K) against the amount loaded (1.25, 5, 10, 15 μg) showed a calculated r value of 0.96, indicating that exposures of the Northern blots were within the linear range.

The expression of hupA was compared to vuuA expression by calculating a ratio of the pixels measured in the Northern blot probed with hupA [Fig. 5(a)] divided by the pixels measured in the Northern blot probed with vuuA [Fig. 5(b)] to control for the effect of added iron source haemin on expression of iron-regulated outer membrane proteins. Reduced expression of hupA (ratio 2.5) in the hupR mutant CML55 was observed, when compared to wild-type (ratio 3.5) or the hupR in trans (ratio 3.1). This data confirms previous observations that hupA is regulated by iron [22] and suggests that HupR is required for wild-type expression of hupA in low-iron media with haemin.

Transcriptional analysis of hupA: wild-type MO6–24 compared to hupR mutant CML55

To further analyse the regulation of transcription of hupA by HupR, Fur, haemin and iron, a hupA–lacZ gene fusion was cloned into a low copy pLAFR3 plasmid. Iron-regulated β-galactosidase activity was seen in both wild-type MO6–24 and hupR mutant CML55 (Fig. 6). Low levels of β-galactosidase activity were observed under high-iron conditions [Fig. 6(a)] or high-iron conditions with haemin (40 μM) added in both wild-type and the hupR mutant strains. Increased levels of β-galactosidase were observed in both wild-type and hupR mutant strains under low-iron conditions. β-galactosidase activity was no longer regulated by iron in the fur mutant CML17 [Fig. 6(a)]. The addition
Galactosidase activity was approximately 30% higher in high-iron conditions. Adding 40 μM haemin at each haemin concentration were compared using Student’s t-test (n = 10). Transcription of *hupA* in wild-type (MO6–24) and mutant (CML55) grown in low-iron with added haemin are compared using Student’s t-test (n = 10).

Figure 6. Regulation of the outer membrane protein HupA in response to iron restriction and increasing concentrations of haemin. Transcription of *hupA* was assayed by using *hupA–lacZ* fusions on plasmid pCML62. β-Galactosidase assays were performed with late-log phase cells (OD600nm 0.8–1.0) grown under high-iron (LB) and low iron (0.1 mM FeSO4, 2,2′-dipyridyl) conditions. Cells of wild-type (MO6–24), *hupR* mutant (CML55) and *fur* mutant (CML17) strains containing pCML62 were tested. Error bars indicate standard deviations. P = 0.01 when the β-galactosidase activities of MO6–24 and CML55 grown in low-iron with added haemin are compared using Student’s t-test (n = 10).

(b) Regulation of the outer membrane protein HupA in response to iron restriction and increasing concentrations of haemin. Transcription of *hupA* was assayed by using *hupA–lacZ* fusions on plasmid pCML62 in wild-type (MO6–24) and *hupR* mutant (CML55) background. β-Galactosidase assays were performed with late-log phase cells grown under low iron (0.1 mM 2,2′-dipyridyl) conditions with haemin concentrations increasing from 0–40 μM. Error bars indicate standard deviations. β-Galactosidase activities of MO6–24 and CML55 at each haemin concentration were compared using Student’s t-test (n = 10).

of haemin to *fur* mutant CML17 did not significantly affect β-galactosidase activity. β-Galactosidase activity was approximately 30% reduced in the wild-type strain when it was grown in low-iron media with 40 μM haemin added, compared to the activity in low-iron media alone (P = 0.01; n = 10; Student’s t-test). The *hupR* mutant (CML55) grown in low-iron media with 40 μM haemin showed over a 50% reduction in β-galactosidase activity compared to CML55 grown in low-iron media alone (P < 0.001; n = 10). β-Galactosidase activity of the *hupR* mutant (CML55) grown in low-iron media with 40 μM haemin was 40% lower than with the wild-type (MO6–24) grown in low-iron media with 40 μM haemin (P = 0.01; n = 10). There was no significant difference in β-galactosidase activity between MO6–24 and CML55 when grown in low-iron media alone. The β-galactosidase activity of the *hupR* mutant grown in low-iron media with haemin added in concentrations ranging from 10–40 μM, was significantly reduced compared to wild-type grown in low-iron media with haemin [P < 0.001; n = 10, for 10–30 μM, P = 0.01 for 10 μM; Fig. 6(b)]. Increasing the amount of haemin added to the media (up to 100 μM) showed a progressive decrease in the β-galactosidase activity in the wild-type vs CML55 to baseline levels seen in high-iron conditions. Adding 40 μM FeSO4 to the media chelated with dipyridyl also decreased the β-galactosidase activity to baseline levels in MO6–24 and CML55. The data from the Northern blot analysis and the *hupA–lacZ* fusion studies suggest that *hupR* may play a role in activating transcription of *hupA*, under iron restrictive conditions in the presence of haemin.

Outer membrane protein analysis: wild-type MO6–24 compared to *hupR* mutant CML55

We compared the outer membrane proteins of wild-type *V. vulnificus* and strain CML55 after growth in high-iron, low-iron and low-iron media supplemented with 20 μM haemin (Fig. 7). The intensity of the bands was examined densitometrically. In both wild-type *V. vulnificus* and CML55, two proteins with apparent molecular sizes of 72 and 77 kDa appeared after growth under low-iron conditions, with no apparent difference in expression of the two proteins between the strains. The 72 and 77 kDa
More than 200 proteins from diverse prokaryotic genera have been identified as members of this family [23]. Many of these proteins regulate the expression of divergently transcribed, linked genes. All proteins in this family have a helix-turn-helix motif near the N-terminus which is involved in DNA binding. HupR has both these characteristics in common with members of the LysR family of transcriptional activators.

LysR type transcriptional regulators usually recognize and bind two different areas near the promoter region of the regulated gene. There is often a recognition sequence located at approximately −65 (with respect to the regulated gene), and an activation sequence near the −35 RNA polymerase binding site. The recognition sequence usually consists of a partially dyadic sequence and a T-N11-A motif. A perfect inverted repeat was also identified near the −35 RNA polymerase binding site, which may be a good candidate for the activation site of hupA.

For many LysR type transcriptional activators, interaction with the activation site (near the −35 RNA polymerase binding site) often requires a coinducer as a prerequisite for transcriptional activation [23]. The data in the present study suggests that HupR is a positive transcription activator of hupA transcription in the presence of haem under iron-limiting conditions (Figs 5–7). Initially, we examined the effect of HupR on hupA expression by introducing a hupR mutation into the chromosome of MO6–24. The resulting mutant, CML55, maintained wild-type expression under both high- and low-iron concentrations. This finding was confirmed by both Northern blot analysis and by expression of a hupA–lacZ fusion [Fig. 5(a), lanes 1–4; Fig. 6(a) and Fig. 7]. These observations led us to suspect that, as with many LysR type transcriptional activators, a coinducer is required for transcriptional activation of the target gene. Under low-iron conditions with added haemin, hupA expression was increased in the wild-type when compared to the hupR mutant CML55. This differential expression was seen in Northern blot analysis (Fig. 5, lanes 7–8), hupA–lacZ fusion analysis (Fig. 6) and in analysis of the outer membrane proteins (Fig. 7).

The haem response of hupA in CML55 was restored to wild-type levels when complemented with hupR in trans (Fig. 5). Usually, LysR transcriptional activators will increase
transcription of target promoters between six- and 200-fold in the presence of a coinducer [23]. The transcriptional activation by HupR in the presence of haem was only two- to three-fold, suggesting that HupR may be a weak activator of transcription; alternatively, the coinducer conditions may not have been optimized for activation.

A LysR family positive transcriptional regulator of an iron-regulated gene has been previously described for *V. cholerae* [27]. HupR shows 20% similarity to IrgB, a protein which regulates the transcription of the *V. cholerae* virulence gene, *irgA*, which encodes an iron-regulated outer membrane protein. *irgB* is unlike *hupR*, in that insertional inactivation of *irgB* leads to the total loss of expression of its target gene, *irgA*. However, like *hupR* and *hupA*, *irgB* and *irgA* are inversely transcribed. The promoters of *irgB* and *irgA* contain a Fur-binding site within their overlapping promoters and hence both are negatively regulated by iron and Fur. In contrast, the promoters of *hupR* and *hupA* are divergent but do not overlap. The promoter of *hupA* contains the Fur-binding site between the −10 and −35 RNA polymerase-binding sites. The location of the Fur-binding site within the *hupA* promoter, but distal from the *hupR* promoter, may explain the experimental observation that *hupA* is strongly negatively regulated by iron, whereas *hupR* is only weakly regulated by iron.

Recently, a two-component signal transduction system was identified that activates expression of the *hmuo* gene of *C. diphtheriae* in response to haem and haemoglobin [21]. The *C. diphtheriae* *hmuo* gene encodes a haem oxygense that is involved in the utilization of haem as an iron source [20]. Transcription of *hmuo* is controlled under a dual regulatory mechanism in which the diphtheria toxin repressor protein (DtxR) and iron repress expression, while haem is needed to activate transcription. A response regulator (*chrA*) and its cognate sensor kinase (*chrS*) was shown to activate transcription from the *hmuo* promoter in a haem-dependent manner [21]. Transcription of the *hmuo* promoter is repressed by DtxR during growth in the presence of iron. The *hmuo* gene is optimally expressed in low-iron environments in the presence of haem. This is similar to what was observed with the regulation of *hupA* by HupR, haem and Fur, with the exception that low-iron conditions alone could activate transcription of *hupA*.

We suggest the following model for the regulation of *hupA* by HupR. Under iron-replete conditions it is known that the Fur repressor binds to Fur boxes within iron-regulated promoters, thus repressing transcription of iron-regulated genes [28–30]. Our data suggest that under iron-replete conditions, even in the presence of haem, the active Fur repressor protein represses transcription of *hupA*, and prevents transcriptional activation via HupR. Under low iron conditions, in the presence of haem, the Fur repressor is inactive and transcription of iron-uptake genes, including *hupA*, is allowed. Under conditions of relative iron restriction, in the presence of haem, the HupR protein, could act as a transcriptional activator of *hupA*. This would serve to increase the relative amounts of the haem receptor compared to other iron uptake mechanisms, thus allowing the bacterial cell preferentially take up haem if it is more readily available than other iron sources.

In our model, a *fur* mutant represents maximal activity of the *hupA* promoter, regardless of the presence of HupR and its coinducer. Consistent with this model, the β-galactosidase activity of the *fur* mutant was much higher than observed for iron-restrictive conditions in both MO6–24 and CML55. It is probable that *hupA* contains a strong promoter, since there is a high degree of homology between the *hupA* −10 and −35 boxes and the *E. coli* RNA polymerase binding consensus sequences.

It is not surprising that this organism has evolved a complex and efficient mechanism of iron sensing and uptake. The ability of *V. vulnificus* to colonize the bloodstream is certainly enhanced by its capacity to sense and respond to both free iron and important host iron sources such as haem and haemoglobin. This mechanism complements the organism’s ability to lyse red blood cells via its haemolysin–cytolysin, since much of the iron released by disrupted erythrocytes is bound to haem or haemoglobin. Responding to these iron-carriers is clearly an advantage in the competition for this limited resource. An efficient response to haem could be an important virulence determinant, and a critical factor in septicemia. *V. vulnificus* causes sepsis in susceptible patients following oral ingestion, with mortality rates exceeding 50%. Future studies, involving the purification of the *V. vulnificus* HupR protein, will examine DNA binding and transcriptional regulation by this protein.
Table 1. Strains and plasmids used in this study

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<tr>
<td>80363</td>
<td>Str&lt;sup&gt;r&lt;/sup&gt;, opaque</td>
<td>(44)</td>
</tr>
<tr>
<td>CML17</td>
<td>80363Δ fur</td>
<td>[44]</td>
</tr>
<tr>
<td>MO6-24</td>
<td>Poly&lt;sup&gt;r&lt;/sup&gt;, opaque</td>
<td>[12]</td>
</tr>
<tr>
<td>CML55</td>
<td>MO6-24, Δ(hupR)</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5&lt;sup&gt;x&lt;/sup&gt;</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 Δ(argF–lacZYA) U169 (Φ60ΔlacZ M15λ&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>[45]</td>
</tr>
<tr>
<td>SY327&lt;sup&gt;λ&lt;/sup&gt;pir</td>
<td>Δ(lac pro) nalA recA56 araD argE(Em) pir R6K</td>
<td>(46)</td>
</tr>
<tr>
<td>SM10&lt;sup&gt;λ&lt;/sup&gt;pir</td>
<td>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu λpir R6K Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>[46]</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC19</td>
<td>Cloning vector, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>pBluescript SK&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Phagemid derived from pUC19; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Strategene</td>
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<tr>
<td>pLAFR3</td>
<td>Cloning vector; Tc</td>
<td>[47]</td>
</tr>
<tr>
<td>pU110</td>
<td>8.9 kbp vector containing promoterless lacZ and phoA genes to allow for generation of gene fusions; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>[48]</td>
</tr>
<tr>
<td>pCML37</td>
<td>hupA clone from V. vulnificus MO6-24 genomic library; 8 kb chromosomal fragment in pBluescript SK&lt;sup&gt;−&lt;/sup&gt;; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>[22]</td>
</tr>
<tr>
<td>pCML38</td>
<td>1.7 kb HindIII V. vulnificus hupA clone in pBluescript SK&lt;sup&gt;−&lt;/sup&gt;; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>[22]</td>
</tr>
<tr>
<td>pCML43</td>
<td>1.5 kb Vsp/I/AflIII hupR clone in HincII site in pUC19; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pCML55</td>
<td>1.5 kb Vsp/I/AflIII hupR clone in pLAFR3; Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pCML46</td>
<td>pCML43 with 443 bp deletion within hupR from PmaCl to MscI; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pCVD442</td>
<td>Positive selection suicide vector, pGP704 with sacB gene inserted in multiple cloning site; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>[36]</td>
</tr>
<tr>
<td>pCML50</td>
<td>1.1 kb SacI/SphI fragment from pCML46 inserted into SacI/SphI site of pCVD442; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pCML48</td>
<td>4.1 kb promoterless lacZ (from pU110) insertion into MscI site of hupA in pCML38, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pCML58</td>
<td>vuuA clone from V. vulnificus MO6-24 genomic library; 3.3 kb chromosomal fragment in pBluescript SK&lt;sup&gt;−&lt;/sup&gt;; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>[25]</td>
</tr>
<tr>
<td>pCML62</td>
<td>hupA–lacZ from pCML48 inserted in multiple cloning site of pLAFR3, Ap&lt;sup&gt;r&lt;/sup&gt;, Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>

Materials and Methods

Bacterial strains and plasmids

Characteristics of the V. vulnificus and E. coli strains and plasmids used in this study are described in Table 1. Subclones are described in Fig. 1.

Media

Strains were routinely grown in LB. All strains were maintained at −70°C in LB media containing 15% glycerol. LB solidified with agar was used for high-iron solid media. Two types of low iron media were used: LB medium with the addition of the iron chelator 2,2′-dipyridyl (Sigma Chemical Co., St Louis, MO, U.S.A.) to a final concentration of 0.1 mM. Haemin was added in some studies to iron-chelated media to a final concentration of 40 μM. LB medium was made iron deficient by the addition of 75 μg/ml of ethylenediamine-di(o-hydroxyphenyl)acetic acid (EDDA), deferred by the method of Rogers [31]. Ampicillin (100 μg/ml), kanamycin (45 μg/ml), polymyxin B (50 U/ml), tetracycline (15 μg/ml for E. coli; 1.5 μg/ml for V. vulnificus) or
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; International Biotechnologies, Inc., New Haven, Conn., U.S.A., 40 μg/ml) was added as appropriate.

**DNA manipulations and cloning**

Standard methods were followed for molecular biological techniques [32]. Oligonucleotides were synthesized at the Huntsman Cancer Center Peptide and DNA facility (University of Utah, U.S.A.). Oligonucleotides were labelled with phage T4 polynucleotide kinase and plasmid DNA fragments were labelled with a random prime kit (Bethesda Research Laboratories Life Technologies, Gaithersburg, MD, U.S.A.). Restriction enzyme-digested genomic and plasmid DNA fragments were resolved through 1.0% agarose gels and DNA was transferred to GeneScreen Plus membranes (Du Pont, NEN Research Products) by the method of Southern [33]. High-stringency hybridizations were performed at 42°C in a buffer containing 1 M NaCl, 1% sodium dodecyl sulfate and 50% formamide. After 6–24 h of hybridization, the membranes were washed according to the manufacturers recommendations and visualized by autoradiography.

**DNA sequencing**

The DNA sequence was determined by the di-deoxy-chain termination method of Sanger et al. [34] on double-stranded DNA plasmid templates by using a Sequenase kit from United States Biochemical Corporation (Cleveland, Ohio, U.S.A.) and by the ABI Prism 377 DNA Sequencer from Applied Biosystems. Synthetic oligonucleotides used as primers for DNA sequencing were synthesized by the Huntsman Cancer Center DNA peptide facility.

**Construction of V. vulnificus hupR deletion**

A hupR deletion was constructed in V. vulnificus by in vivo marker exchange as described [35]. 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 [36]. The 1.5 kb VspI/AflIII fragment of pCML37 (containing hupR gene) was subcloned in pUC19 and designated pCML43; a 443 bp PmaCl/BalI fragment internal to V. vulnificus hupR was deleted by digestion and re-ligated to yield pCML46. The 1.1 kbp SacI/SphI fragment of pCML46 was ligated into SacI–SphI digested pCVD442, yielding pCML50. In vivo marker exchange was used to replace the chromosomal copy of hupR in V. vulnificus with the internal deleted copy in pCML50 without any remaining integrated plasmid sequences, as described [35, 36] to generate strain CML55.

**Construction of hupA–lacZ fusions**

The promoterless lacZ gene from plasmid pUJ10 contained on a 4.1 kb Smal/NotI fragment was inserted into the MscI site in hupA on pCML38 by ligation and designated pCML48. The hupA–lacZ fusion was subcloned on the lower copy plasmid, pLAFR3 by ligation into the EcoRI site on the polylinker (pCML62).

**Utilization of iron sources**

The utilization of iron sources by V. vulnificus was assayed by the procedure by Simpson and Oliver [37]. Human holotransferrin (Sigma) solubilized in PBS was determined to have an iron saturation of 99% by the Ferrozine assay for Fe [38] performed on a Hitachi 717 Automatic Analyzer (Boehringer Mannheim Corp., Indianapolis, U.S.A.). Haemin (Sigma) was solubilized in 10 mM NaOH and haemoglobin was solubilized in PBS.

**Assays**

β-galactosidase assays were performed on mid-log-phase cultures [39] on strains transformed with pCML62. V. vulnificus strains were grown in LB with 40 μM FeSO₄, LB with 0.1 mM 2,2’-dipyridyl or LB with 0.1 mM 2,2’-dipyridyl with haemin added to a final concentration of 40 μM.

**RNA analysis**

RNAs from cultures grown under high-iron conditions (LB medium), low-iron conditions (LB medium containing 2,2’-dipyridyl) and low-iron conditions with added haemin were prepared
using Trizol reagent, according to the manufacturer’s protocol (Bethesda Research Laboratories Life Technologies). Northern (RNA) blot analysis was performed by using standard molecular biological techniques [32]; 10 μg of RNA, as calculated from the optical density at 260 nm, were loaded into all of the lanes. The internal 1.17 kbp BglII–HindIII fragment of the V. vulnificus hupA gene was used as the probe for Northern blots examining hupA regulation. The internal 595 bp BglII–EcpRV fragment of the V. vulnificus hupR gene was used as the probe for Northern blots examining hupR regulation. The internal 1.3 kbp MscI–NruI fragment of the V. vulnificus vvuaA gene was used as a probe in examining vvuaA regulation. Primer extension was performed on RNAs from cultures grown under low-iron conditions using a Promega primer extension kit according to the manufacturer’s instructions (Promega, Madison, WI, U.S.A.). End-labelled oligonucleotides were used as probes for Northern blots used in confirming the start site of transcription for hupR.

Preparation and analysis of outer membrane proteins

Enriched outer membrane proteins were prepared by using previously described procedures [40] from cells grown to late logarithmic phase in LB medium with and without added 2,2′-dipyridyl. Haemin was also added in some studies to iron-chelated media to a final concentration of 20 μM. The outer membrane proteins were separated on SDS-PAGE and were stained with Coomassie blue, as described [41].

DNA and protein data base searches

The National Center for Biotechnology Information Services were used to consult the SwissPROT, GenBank and EMBL databases with the BLAST algorithm [42, 43].

Statistics

β-galactosidase units were compared using the Students t-test.

Nucleotide sequence accession number

The GenBank accession number for the sequence presented in this article is AFO47484.

Acknowledgements

We gratefully acknowledge Bob Schackman of the Huntsman Cancer Institute for providing synthetic oligonucleotides (NCI CA42014). This work was supported by Public Health Service grant AI40067 from the National Institute of Allergy and Infectious Diseases to C. M. L.

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AUTHOR QUERIES

i) Note: heme changed to haem throughout to bring in line with journal style.

ii) Please provide address of "Applied Biosystems" (Materials and Methods). M/S p.16.

iii) Please provide address of "Du Pont, NEN Research Products" (Materials and Methods).
     M/S p.15.

iv) Please indicate which is correct: "Huntsman Cancer Center Peptide and DNA facility"
    or "Huntsman Cancer Center DNA peptide facility". M/S pp.15 and 16.

v) M/S p.22. Please supply pages cited for ref. 32.

vi) M/S p.23. Please supply pages cited for ref. 39 and check.