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

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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,

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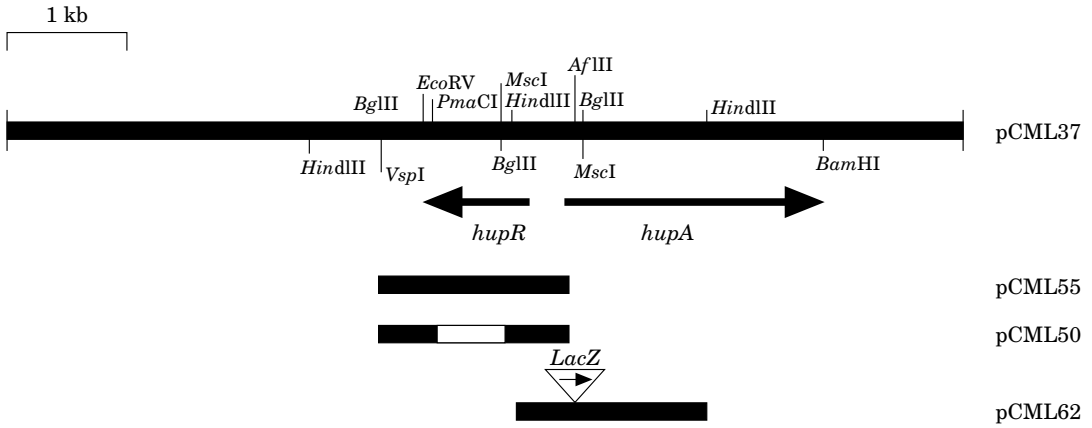

Figure 1. Endonuclease restriction map of *hupA*, *hupR* and flanking DNA in plasmid pCML37. Shown below the restriction map are the inserts present in the various subclones derived from pCML37. Plasmid pCML50 contains the *Vsp*I/*Afl*III insert from pCML55 with an internal deletion of *hupR* DNA from the *Pma*CI site to the *Msc*I site, indicated by the open bar. Arrows indicate direction of transcription of the listed genes.

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

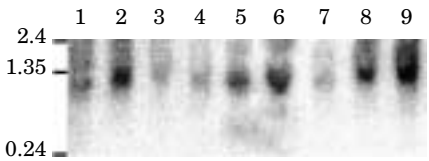

Figure 2. Northern blot analysis of RNA prepared from *V. vulnificus* MO6–24 after growth in high-iron medium (lane 1) and low-iron medium (lane 2), from *V. vulnificus* 80363 after growth in high-iron medium (lane 3) and low-iron medium (lane 4) and *V. vulnificus* CML17 after growth in high-iron medium (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 *Bgl*II-*Eco*RV fragment internal to *hupR*. The positions of single-stranded RNA molecular weight markers (in kb) are indicated on the left.

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 K) 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 base 198 of the sequence in Fig. 3 was identified (data not shown). Potential −35 and −10 boxes are identified upstream of the transcriptional start site.

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 presenteed 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 pI 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).

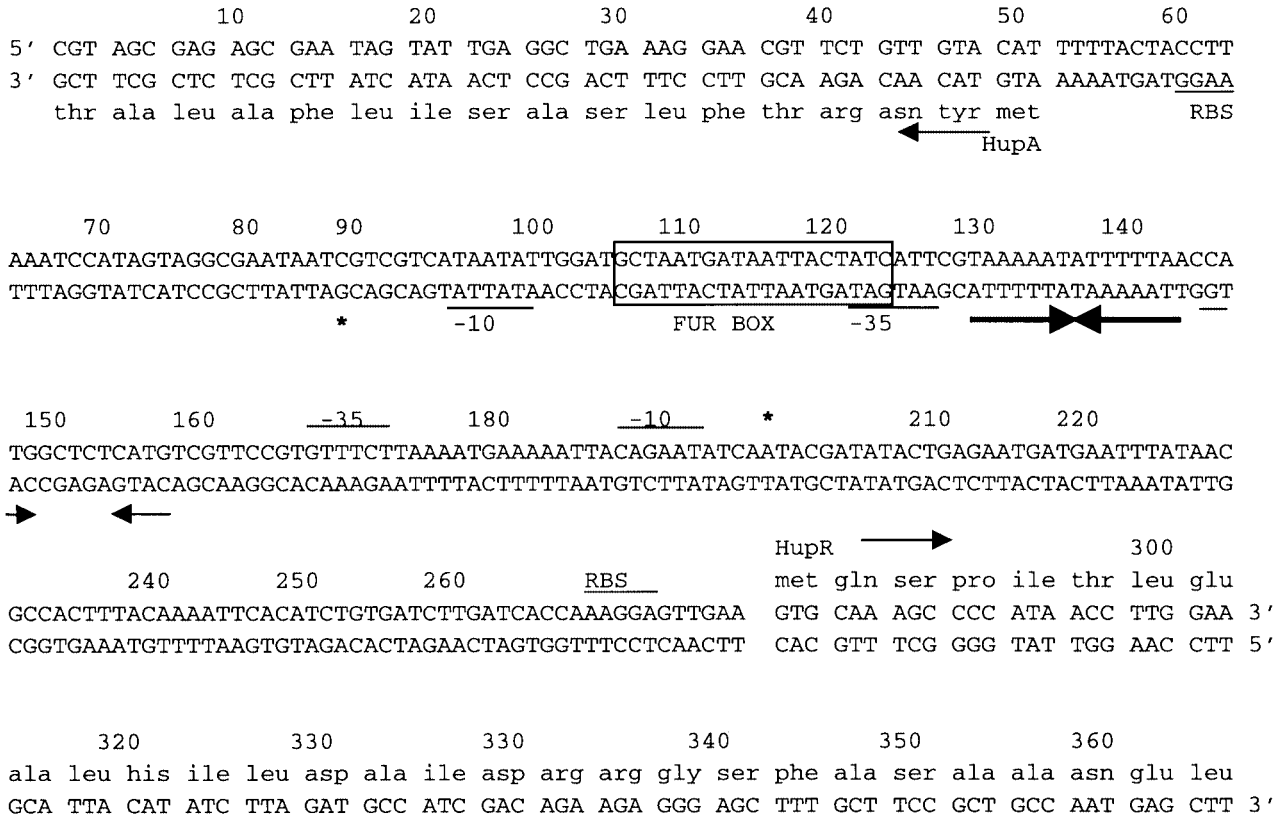

Figure 3. Partial nucleotide sequence of *V. vulnificus hupR*, including the detail of the promoters of *hupR* and *hupA*. The promoter of *hupR* (−35, −10), the approximate start site of transcription (*). The ribosomal-binding site (RBS) and the deduced amino acid sequence are indicated on the upper 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-N_{11}-A motif. Binding also often involves symmetrical guanine bases in the two dyad arms. LysR type transcriptiion 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].

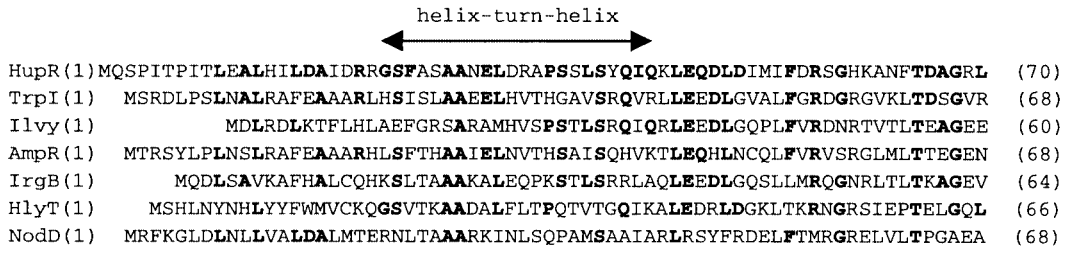

Figure 4. Homology between the amino terminus of HupR and several members of the LysR family of positive transcriptional activators. Amino acids are identified in single letter code and identical residues are in bold text. The conserved helix-turn-helix domain of these proteins is indicated above the sequences.

A 13 bp interrupted inverted repeat sequence (3′GTAC-N_5GTACA5′; 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-N_{11}-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, $FeSO_4$ 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 5 µl 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; $FeSO_4$, 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 haemin). RNA from each sample (10 µg) was loaded in each lane. The blot was probed with the *Bgl*II-*Hin*dIII 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 *Bgl*II/*Eco*RV fragment contained on 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

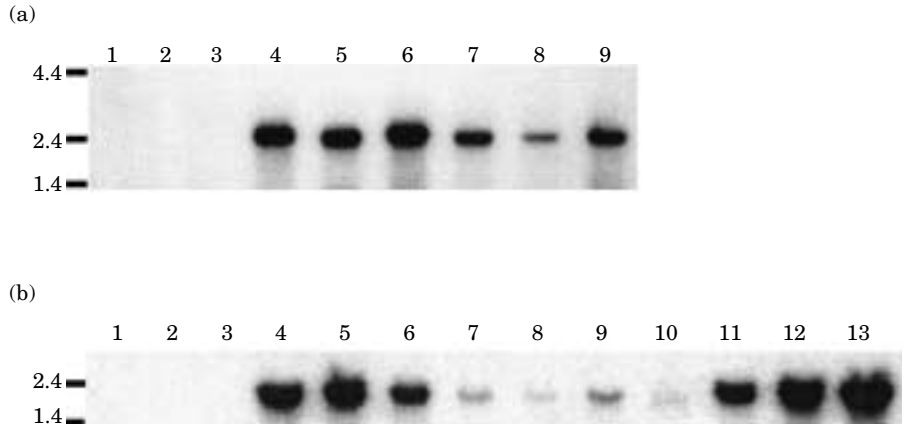

Figure 5. (a) Northern blot analysis of RNA from MO6–24, CML55 and CML55 (pCML55) probed with a DBA fragment internal to *hupA*. Lane 1, MO6–24 grown in high-iron medium; lane 2, CML55 high-iron medium; lane 3, CML55 (pCML55) high-iron medium; lane 4, MO6–24 low-iron medium; lane 5, CML55 low-iron medium; 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

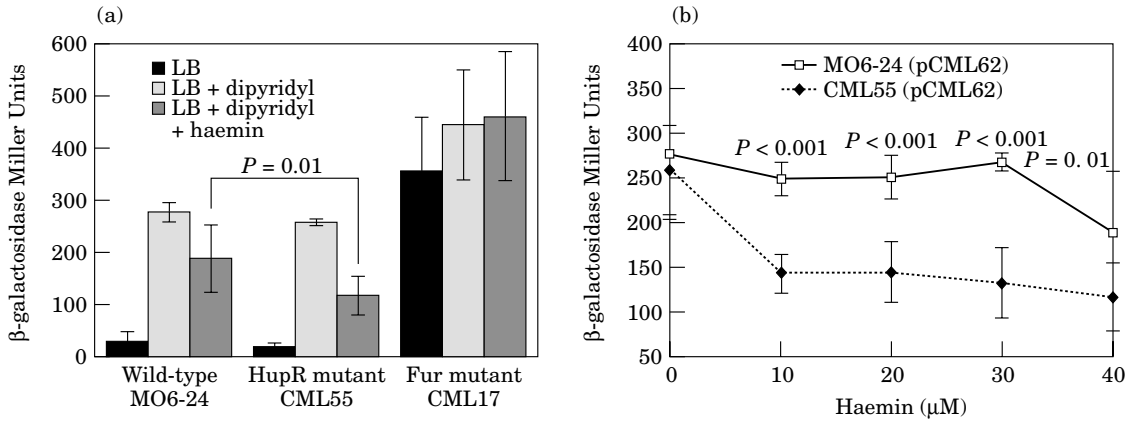

Figure 6. Regulation of the outer membrane protein HupA in response to iron restriction with and without added haemin. Transcription of *hupA* was assayed by using *hupA–lacZ* fusions on plasmid pCML62. β-Galactosidase assays were performed with late-log phase cells (OD_{600nm} 0.8–1.0) grown under high-(LB) iron, low iron (0.1 mM, 2,2′-dipyridyl) or low iron with added haemin (0.1 mM, 2,2′-dipyridyl plus 40 μM haemin) 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 haaemin are compared using Students *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 Students *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; Students *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 $FeSO_4$ 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

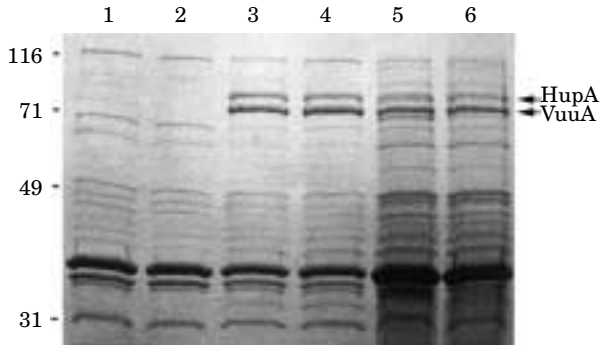

Figure 7. SDS-PAGE of outer membrane proteins. Lane 1, wild-type *V. vulnificus* grown in high-iron medium; lane 2, CML55 high-iron medium; lane 3, MO6–24 low-iron medium; lane 4, CML55 low-iron medium; lane 5, MO6–24 low-iron medium with 20 μM haemin; lane 6, CML55 low-iron medium with 20 μM haemin. The arrows indicate the position of the haem receptor, HupA, and the vulnibactin receptor VuuA.

proteins have been observed to correspond to the vulnibactin receptor (VuuA) [25] and the haem uptake receptor (HupA) [22], respectively. Mutant CML55, however, showed reduced expression of the haem receptor (0.91 K pixels) after growth under low-iron conditions supplemented with haemin, when compared to wild-type *V. vulnificus* (1.44 K pixels). There was no significant difference between the expression of the vulnibactin receptor in the two strains MO6–24 (2.2 K pixels) and CML55 (2.0 K pixels) after growth under low-iron conditions supplemented with haemin.

Discussion

The expression of many iron uptake genes is regulated by the concentration of iron in the environment, with increased expression occurring under low-iron conditions. We have previously characterized and cloned the *hupA* haem receptor in *V. vulnificus* [22]. Transcription of *hupA* was negatively regulated by iron, its promoter contained a 19 bp dyad symmetric sequence homologous to the *E. coli* Fur-binding site. Here, a gene upstream of *hupA*, called *hupR*, was discovered in reverse orientation to *hupA*. The deduced amino acid sequence of HupR shares homology with the LysR family of bacterial transcriptional activator proteins (Fig. 4). 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 that has a conserved T-N_{11}-A motif [26]. The −65 region of *hupA* has an interrupted dyadic sequence and a T-N_{11}-A motif. A perfected 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 oxygenase 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 septicaemia. *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

Strain or plasmid	Relevant characteristic(s)	Reference or source
V. vulnificus strains		
80363	Str^r, opaque	(44]
CML17	80363 Δ(*fur*)	[44]
MO6–24	Poly^r, opaque	[12]
CML55	MO6–24, Δ(*hupR*)	This study
E. coli strains		
DH5α	F$^-$*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF–lacZYA*) *U169* (Φ60Δ*lacZ M15*λ$^-$	45]
SY327λ*pir*	Δ(*lac pro*) *nalA recA56 araD argE*(Am)λ*pir* R6K	(46]
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir* R6K Km^r	[46]
Plasmids		
pUC19	Cloning vector, Ap^r	Laboratory stock
pBluescript SK$^-$	Phagemid derived from pUC19; Ap^r	Strategene
pLAFR3	Cloning vector; Tc	[47]
pUJ10	8.9 kbp vdctor containing promoterless *lacZ* and *phoA* genes to allow for generation of gene fusions; Ap^r	[48]
pCML37	*hupA* clone from *V. vulnificus* MO6–24 genomic library; 8 kbp chromosomal fragment in pBluescript SK$^-$; Ap^r	[22]
pCML38	1.7 kbp *Hin*dIII *V. vulnificus hupA* clone in pBluescript SK$^-$; Ap^r	[22]
pCML43	1.5 kbp *Vsp*I/*Afl*III *hupR* clone in *Hin*cII site in pUC19; Ap^r	This study
pCML55	1.5 kbp *Vsp*I/*Afl*III *hupR* clone in pLAFR3; Tc^r	This study
pCML46	pCML43 with 443 bp deletion within *hupR* from *Pma*CI to *Msc*I; Ap^r	This study
pCVD442	Positive selection suicide vector, pGP704 with *sacB* gene inserted in multiple cloning site; Ap^r	[36]
pCML50	1.1 kbp *Sac*I/*Sph*I fragment from pCML 46 inserted into *Sac*I/*Sph*I site of pCVD442; Ap^r	This study
pCML48	4.1 kbp promoterless *lacZ* (from pUJ10) insertion into *Msc*I site of *hupA* in pCML38, Ap^r	This study
pCML58	*vuuA* clone from *V. vulnificus* MO6–24 genomic library; 3.3 kbp chromosomal fragment in pBluescript SK$^-$; Ap^r	[25]
pCML62	*hupA–lacZ* from pCML48 inserted in multiple cloning site of pLAFR3, Ap^r, Tc^r	This study

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), deferated 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 dideoxy-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 *Vsp*I/*Afl*III fragment of pCML37 (containing *hupR* gene) was subcloned in pUC19 and designated pCML43; a 443 bp *Pma*CI/*Bal*I fragment internal to *V. vulnificus hupR* was deleted by digestion and re-ligated to yield pCML46. The 1.1 kbp *Sac*I/*Sph*I fragment of pCML46 was ligated into *Sac*I–*Sph*I 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 kbp *Sma*I/*Not*I fragment was inserted into the *Msc*I 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 *Eco*RI 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 $FeSO_4$, 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 manufacturers 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 *Bgl*II–*Hin*dIII fragment of the *V. vulnificus hupA* gene was used as the probe for Northern blots examining *hupA* regulation. The internal 595 bp *Bgl*II–*Ecp*RV fragment of the *V. vulnificus hupR* gene was used as the probe for Northern blots examining *hupR* regulation. The internal 1.3 kbp *Msc*I–*Nru*I fragment of the *V. vulnificus vuuA* gene was used as a probe in examining *vuuA* regulation. Primer extension was performed on RNAs from cultures grown under low-iron conditions using a Promega primer extension kit according to the manufacturers 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*. Northern gels were scanned densitometrically and digitized using NIH image Version 1.62 freeware software program (National Institutes of Health, U.S.A.). Densitometric values were expressed as pixels.

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.

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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.
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