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Identification and characterization of a heme periplasmic-binding protein in *Haemophilus ducreyi*

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Abstract *Haemophilus ducreyi*, a gram-negative and heme-dependent bacterium, is the causative agent of chancroid, a genital ulcer sexually transmitted infection. Heme acquisition in *H. ducreyi* proceeds via a receptor mediated process in which the initial event involves binding of hemoglobin and heme to their cognate outer membrane proteins, HgbA and TdhA, respectively. Following this specific interaction, the fate of the periplasmic deposited heme is unclear. Using protein expression profiling of the *H. ducreyi* periplasmic proteome, a periplasmic-binding protein, termed hHbp, was identified whose expression was enhanced under heme-limited conditions. The gene encoding this protein was situated in a locus displaying genetic characteristics of an ABC transporter. The purified protein bound heme in a dose-dependent and saturable manner and this binding was specifically competitively inhibited by heme. The *hhbp* gene functionally complemented an *Escherichia coli* heme uptake mutant. Expression of the heme periplasmic-binding protein was detected in a limited survey of *H. ducreyi* and *H. influenzae* clinical strains. These results indicate that the passage of heme into the cytoplasm of *H. ducreyi* involves a heme dedicated ABC transporter.

Keywords *Haemophilus ducreyi* · Chancroid · Heme · ABC transporter

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Introduction

The cellular phospholipid envelope presents a formidable barrier to the access of nutrients essential to maintain the catalytic and replicative cycle of the cell. In prokaryotes and eukayotes, the transit of molecules into these membrane enclosed compartments is accomplished by a sophisticated transport machinery, termed an ATP-binding cassette (ABC) transporter. This protein complex couples ATP hydrolysis with substrate translocation across biological membranes (Ames 1986; Higgins 1992; Doige and Ames 1993; Schneider and Hunke 1998). The binding-protein dependent transporters of Gram-negative bacteria are the best characterized members of this superfamily. An auxiliary component, a periplasmic-binding protein, is the major determinant in conferring substrate specificity (Ames 1986; Higgins 1992; Doige and Ames 1993; Quiocho and Ledvina

1996; Ehrmann et al. 1998). The genes encoding the transporter are arranged as a single transcriptional unit, although apparent exceptions exist in which the periplasmic binding protein genes are unlinked from their cognate permease genes (Fleischmann et al. 1995; Saurin and Dassa 1996; Linton and Higgins 1998).

Heme serves as a prosthetic group in a variety of biological processes and as a source of iron. As free heme is toxic, a consequence of the catalytic generation of ROS that promotes peroxidative damage to a variety of biomolecules (Miller and Britigan 1997), the bacterial systems to acquire this metalloporphyrin involve a series of protein escorts whereby a heme dedicated ABC transporter plays a pivotal role in ferrying heme into the cytoplasm (Stojiljkovic and Perkins-Balding 2002). The participation of protein chaperons is ubiquitous, as heme trafficking in eukaryotes also appears to obey this fundamental necessity (Quigley et al. 2004).

Haemophilus ducreyi, a Gram-negative heme obligate coccobacillus, is the causative agent of chancroid, a sexually transmitted genital ulcer disease (Albritton 1989; Morse 1989). The mechanism of heme acquisition in *H. ducreyi* remains unclear. The organism expresses outer membrane proteins that specifically bind either to hemoglobin (Elkins 1995; Elkins et al. 1995; Stevens et al. 1996) or to heme (Thomas et al. 1998), but the detailed molecular events subsequent to these surface interactions have yet to be fully elucidated. Although a heme ABC transporter would be predicted to shuttle the periplasmic deposited heme into the cytoplasm, the identification of this system has proven elusive.

In this study we have applied a proteomic approach to isolate a heme periplasmic-binding protein, designated hHbp, in *H. ducreyi*. As the corresponding gene is situated in a locus displaying nucleotide sequence homology to ATP cassette binding proteins, we propose that this periplasmic-binding protein is a constituent of a *H. ducreyi* heme permease.

Materials and methods

Bacterial strains, plasmids and growth conditions

Bacteria used in this study are listed in Table 1. *Escherichia coli* strains FB827 *dppA*::*Cm mppA*::*Km* (pAM238-hasR), and FB827 *dppF*::*Km* (pAM238-hasR)(pTRC99-dppABCDF) have been described previously (Létoffé et al. 2006). Plasmid pET151-hHbp is described in this paper. *H. ducreyi* strains were grown on chocolate agar (CA) containing GC medium base (Difco/Becton-Dickinson, Sparks, Maryland), 1% (wt./vol.) bovine hemoglobin (BBL Microbiological Systems, Cockeysville, Maryland), 1% (vol./vol.) IsoVitaleX (BBL Microbiological Systems) at 33°C in an atmosphere of 5% CO_2. Plates containing heme and the iron chelator desferrioxamine were prepared without bovine hemoglobin enrichment. Heme stock solutions were made by dissolving bovine hemin chloride in 0.1 N NaOH and were used without further sterilization. Desferoxamine (Ciba Pharmaceutical Co., Summit, New Jersey) was dissolved in water at a concentration of 10 mM and sterilized using 0.75 mm filters (Nalge Nunc International, Rochester, New York). Protoporphyrin IX (PPIX (Frontier Scientific, Logan, Utah) was dissolved in 10% (vol./vol.) ethanol. *E. coli* and *Yersinia enterocolitica* strains were maintained at 35°C on Luria-Bertani (LB) agar plates [LB broth (Difco/Becton-Dickinson), 1.5% (wt./vol.) Bacto Agar], M63, M63* (without added iron salt), or grown in LB broth with vigorous shaking at 225 rpm. When needed, 2,2′ dipyridil was added at a concentration of 100 μM to M63* to chelate excess iron. *H. influenzae* strains were grown at 35°C in an atmosphere containing 5% CO_2. When necessary, antibiotics were added at the following concentrations (μg/ml): ampicillin, 100; kanamycin, 25; spectinomycin, 50; tetracycline, 10; and chloramphenicol, 15.

Chemicals and reagents

All chemicals were purchased from Sigma Chemical Co., St. Louis, Missouri, unless otherwise specified.

Isolation of periplasmic, cytoplasmic and outer membrane fractions

Haemophilus ducreyi 35000 was harvested from bacterial lawns on plates grown under heme restricted (7.7 μM heme and 100 μM desferrioxamine) and heme replete (77 μM heme and 100 μM desferrioxamine) conditions. To exhaust intracellular heme and iron stores, bacteria were sequentially subcultured three times on the heme and iron depleted agar. The

Table 1 Bacterial strains

Strains	Relevant characteristics	Source
H. ducreyi		
35000	Clinical isolate; Winnipeg, Canada	Lee (1991)
J1159	Clinical isolate; Nairobi, Kenya (1986–1987); serotype E; LOS Class 6	Lee (1991)
G29677	Clinical isolate; Nairobi, Kenya (1986–1987); LOS Class 4	W. Cameron
C148	Clinical isolate; Nairobi, Kenya (1981); serotype A; LOS Class 1	Lee (1991)
PPC 263/1293	Clinical isolate; Nairobi, Kenya (1986–1987); serotype E; LOS Class 3	W. Cameron
V1157	Clinical isolate; Seattle, USA; serotype C; LOS Class 5	Lee (1991)
RO-27	Clinical isolate; Nairobi, Kenya (1986–1987); LOS Class 2	W. Cameron
K10159	Clinical isolate; Nairobi, Kenya (1986–1987); LOS Class 1	W. Cameron
R0-12	Clinical isolate; Nairobi, Kenya (1986–1987); LOS Class 2	W. Cameron
36-F-2	Pasteur Institute, Paris, France; LOS Class 3	Lee (1991)
PPC358/1315	Nairobi, Kenya (1986–1987); serotype D; LOS Class 7	W. Cameron
H. influenzae		
70824	Non-typeable; Biotype III; blood culture clinical isolate	F. Chan
15631	Non-typeable; Otitis Media clinical isolate	F. Chan
HI-38	Non-typeable; Otitis Media clinical isolate	F. Chan
51944	Non-typeable; Biotype III; blood culture clinical isolate	F. Chan
E. coli		
One Shot® TOP10	*F⁻ mcrA* Δ(*mrr-hsdRMS-mcrBC*)_80*lacZ*Δ*M15* Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (*str*^{R}) *endA1 nupG*	Invitrogen
BL21 Star (DE3)	*F⁻ ompT hsdS*$_B$ ($r_B^- M_B^-$) *gal dcm rne131 (DE3)*	Invitrogen

periplasm was isolated by chloroform extraction (Ames et al. 1984). The cytoplasmic fraction was collected as previously described (Ames et al. 1984). Bacterial outer membranes were prepared by Sarkosyl extraction (Scheffler et al. 2003).

SDS–PAGE and Western blot analysis

One-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was accomplished as described previously (Negari et al. 2008). Gels were stained in a laminar flow hood with either Coomassie, RapidStain (G Bioscience, St. Louis, Missouri), silver, or SYPRO Ruby. Western immunoblot analysis was performed as previously described (Negari et al. 2008). Membranes were probed with rabbit polyclonal antiserum raised against either hHbp in a dilution of 1:8,000, *H. ducreyi* 35000 SodC (Negari et al. 2008) in a dilution of 1:1000, or *H. ducreyi* 35000 outer membranes (Scheffler et al. 2003) in a dilution of 1:1,000, followed by the addition of a 1:10,000 dilution of goat anti-rabbit immunoglobulin horse radish peroxidase conjugated secondary antibody (BioSource, Camarillo, California) solution.

Two-dimensional gel electrophoresis

A modified version of the ReadyStrip IPG strip protocol (BioRad Laboratories, Hercules, California) was used for two-dimensional (2-D) gel electrophoresis. Protein desalting spin columns (Pierce, Rockfort, Illinois) were used to desalt the periplasmic extracts according to the manufacturer's instructions prior to IPG strip rehydration. The desalted periplasmic extracts were quantified using the BCA protein assay reagent (Pierce) and stored at −20°C. Ten micrograms of desalted *H. ducreyi* 35000 periplasmic proteins were used to rehydrate the 7 cm ReadyStrip™ IPG strips pH 3–10 (BioRad). The strips were focused at 20°C with a maximum current of 50 μA per IPG strip using the Protean IEF cell (BioRad) in a two step gradient program comprising 250 V for 15 min followed by 5000 V until 28,000 Volt-h were attained. The gel was equilibrated in a reduction buffer [26.5 mM Tris–HCl, 35.25 mM Tris Base,

2.7% (wt./vol.) SDS, 2.5% (vol./vol.) glycerol, 2% (vol./bol.) DTT] and in an alkylation buffer [26.5 mM Tris–HCl, 35.25 mM Tris Base, 0.5% (wt./vol.) SDS, 2.5% (vol./vol.) glycerol, 2.5% (wt./vol.) iodoacetamide] for 15 min each at 37°C. Proteins were separated in the second dimension by SDS–PAGE. The 2D gels were washed twice at 30 min each in fixer solution [50% (vol./vol.) ethanol, 5% (vol./vol.) acetic acid] and stained overnight with SYPRO Ruby. Differences in net intensities were determined using 2-D analysis software (PDQuest; BioRad). Criteria for statistical significance of spot abundance changes in nonparametric Student's *t*-tests were set at a P value <0.05 (ratio >1.5, heme restricted/heme sufficient growth).

Protein spots of interest were excised from the gel and the candidate protein spots were sent to the Ontario Genomics Innovation Center (OGIC; Ottawa, ON) for identification by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF–MS). The peptide mass maps were analyzed by Mascot (Matrix Science Inc., Boston, Massachusetts) to identify the protein spots. A 95% confidence level threshold was used for Mascot protein scores.

Production of polyclonal antibody reactive against H. ducreyi 35000 hHbp

Rabbit IgG polyclonal antibody against hHbp was produced by immunization with metal chelate affinity-purified hHbp and Gebru adjuvant (Gebru Biotechnik, Gaiberg, Germany) using a described method (Negari et al. 2008). One week after the final injection, polyclonal antisera specific against recombinant hHbp was detected by Western immunoblot (data not shown). No hHbp-specific immunoreactive bands were present when the recombinant protein was reacted either with pre-immune sera or with secondary antibody alone. Serum was collected and aliquots were stored at −70°C.

Cloning and genetic techniques

Restriction endonuclease digestions, ligations, transformations, and agarose gel electrophoresis were performed using standard methods. Plasmid DNA was extracted using the PureLink™ HiPure Mini Plasmid DNA Purification kit (Invitrogen, Carlsbad, California). Amplified gene products and plasmid constructs were verified by fluorescent DNA sequencing using the Applied Biosystems 3730 Analyzer.

Expression of *H. ducreyi* 35000 hHbp

The pET151/D-TOPO® kit (Invitrogen) was used to express the recombinant hHbp protein with a N-terminal fusion tag containing the V5 epitope and polyhistidyl 6xHis region. *H. ducreyi* 35000 genomic DNA was extracted using the QiAmp DNA minikit (Qiagen, Mississauga, Ontario) according to the instructions supplied by the manufacturer and was used as template for PCR amplification of the hHbp gene. The forward and reverse primers pET151-hHBPFOR: 5′-CACCATGAATCTTTCCTTTCTAA; pET151-hHBPREV: 5′-GGATGCTGTAGCTTGTG TTATATTG (Invitrogen), designed using Whitehead Institute for Biomedical Research web-based primer selection interface (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), were incorporated in the following PCR reaction: 0.625 U PfxUltima™ proofreading DNA polymerase, 1× PfxUltima™ PCR buffer, 1 mM dNTPs, pH 8.0 at 0.25 mM each, 0.4 μM primers at 0.2 μM each, and 2 μl *H. ducreyi* genomic DNA as template. The TOPO® cloning reaction was comprised of the 940 bp PCR product at a molar ratio of 1:4 to TOPO® vector and chemical transformation into *E. coli* TOP10 cells was completed as per the manufacturer's instructions.

Transformants were selected from LB ampicillin agar plates and analyzed by colony PCR. Plasmids encoding hHBP were introduced into BL21 Star™ (DE3) One Shot® *E. coli* cells by transformation. Expression of the recombinant fusion protein was achieved by induction with isopropylthio-β-galactoside (IPTG; Invitrogen) at a final concentration of 1 mM added at mid-log (OD_{600} 0.5–0.8) of growth.

Purification of *H. ducreyi* 35000 hHbp

The polyhistidine-containing recombinant hHbp was purified by immobilized metal affinity chromatography on a nickel-nitrilotriacetic acid (NTA) resin (Invitrogen) from IPTG induced BL21 Star™ (DE3) *E. coli* denatured whole cell lysates containing the pET151-hHBP vector. In the final elution step, the protein eluted optimally in the 100 and 150 mM imidazole fractions as verified by SDS–PAGE. These fractions were then buffer exchanged with 1×

purification buffer (50 mM NaH_2PO_4, pH 8.0, 0.5 M NaCl) and concentrated using Amicon Ultra centrifugal filter devices (Millipore, Billerica, Massachusetts). The purified hHbp protein was resolved by 1-dimensional SDS–PAGE and a colloidal blue stained band corresponding to a predicted size of 31 kDa was excised from the gel and analyzed by MALDI tandem mass spectrometry.

Heme agarose binding

For the direct binding assay, increasing amounts of purified hHbp from 500 ng to 10 μg were added to 20 μl of heme agarose beads (≥4 μmol hemin/ml; Sigma) and incubated with gentle agitation on a Labquake Shaker Rotisserie (Barnstead/Thermolyne, Dubuque, Iowa) at 20°C for 1 h in 500 μl of binding buffer (25 mM Tris–HCl pH 8.0, 100 mM NaCl). Samples were centrifuged, washed three times in binding buffer, and bound protein was eluted from the affinity resin by boiling in SDS sample buffer [125 mM Tris, 20% (vol./vol.) glycerol, 4% (wt./vol.) SDS, 10% (vol./vol.) 2-mercaptoethanol, 0.002% (wt./vol.) bromophenol blue] for 5 min prior to separation on SDS–PAGE. In the competitive binding experiments, 10 μg of purified hHbp protein was incubated with increasing concentrations of the competing ligand at 20°C for 1 h in a final volume of 200 μl of PBS prior to precipitation with hemin-agarose beads suspended in 300 μl of binding buffer. Samples were subjected to the heme affinity chromatography protocol as described above. A purified N-terminal 6xHis tagged recombinant outer membrane lipoprotein, rLipL32, from *Leptospira interrogans*, expressed in *E. coli* BL21 Star™ (DE3) (kindly provided by M. Lin, Canadian Food Inspection Agency), served as a negative control in the heme agarose binding experiments.

Heme detection by enhanced chemiluminescence

Purified hHbp protein samples were incubated with increasing concentrations of heme from 10^{-4} to 10^{-7} M for 30 min at room temperature and the mixtures were resolved on non-denaturing PAGE at 4°C (Létoffé et al. 2006). Separated proteins were transferred onto PVDF membranes and heme-protein complexes were detected by enhanced chemiluminescence (ECL+; Amersham Pharmacia Biotech Inc., Piscataway, New Jersey) (Vargas et al. 1993).

Peptide inhibition of heme binding to hHbp was assessed by incubating purified hHbp with heme alone (10^{-4} M) or with a mixture of heme (10^{-4} M), peptone (at a final concentration of 7%) and tryptone (at a final concentration of 7%) at room temperature for 30 min. Mixtures were separated by PAGE and heme was visualized by ECL as described above.

Complementation assays

The presence of the *Serratia marcescens* outer membrane heme receptor HasR permits the recombinant *E. coli* strain FB827 (pAM238-hasR) to use heme as the sole iron source (Létoffé et al. 2006). Once deposited in the periplasmic space, the tetrapyrrole is subsequently conveyed into the cytoplasm by the Dpp oligopeptide ABC transporter *dppBCDF*, in conjunction with either of two periplasmic-binding proteins, MppA or DppA (Létoffé et al. 2006). Plasmid constructs pET151-hHbp and pET151 were introduced by transformation into an *E. coli* FB827 (pAM238-hasR) derivative bearing the *dppA*:::*Km mppA*::*Cm* double mutation, which inactivates the DPP-heme permease (Létoffé et al. 2006). The ability of the constructs to restore the heme competency of the mutant was assayed using a Steer's replicator to deliver $\sim 10^4$ c.f.u. at point of contact onto iron-restricted M63* agar supplemented with hemoglobin and 1 mM IPTG to induce protein expression from the *lac* promoter. This method allowed an individual transformed strain to be inoculated six times onto each plate. Experiments were performed in triplicate.

Results

Analysis of *H. ducreyi* periplasmic protein fractions by 2-D gel electrophoresis

The strategy used to identify the heme-dedicated periplasmic binding protein in *H. ducreyi* 35000 capitalized on the observation that the expression of an ABC transporter is enhanced under ligand-restrictive conditions (Ames 1986; Higgins 1992; Doige and Ames 1993; Schneider and Hunke 1998). Accordingly, 2-D gel profiles of periplasmic proteins

extracted from cells grown under heme-limiting (15 μg/ml heme and 100 μM desferoxamine) and heme-replete (100 μg/ml heme and 100 μM desferoxamine) conditions were compared. The additional imposition of iron limitation accentuates the heme deficiency as the exogenously supplied porphyrin not only provides the obligatory heme, but also serves as the sole source of iron for the heme dependent bacterium. Periplasmic extracts from each of the two conditions were independently isolated in triplicate to control for gel to gel variation. The resulting six extracts were simultaneously co-resolved in duplicate across a total of 12 2-D gels producing six pairwise comparisons. A marked difference in protein expression was seen (Fig. 1a, b). The proteome pattern was highly reproducible with regard to the total number of spots detected and their relative positions and intensities. No significant inter- or intra-sample variations were observed as replicates from the same sample, or from separate preparations were identical (data not shown).

To ensure that each sample was enriched for periplasmic proteins, Western immunoblots of periplasmic cell fractions were probed with an antibody against *H. ducreyi* 35000 SodC, a protein localized in the periplasmic space (San Mateo et al. 1998; Pacello et al. 2001). An immunoreactive band representing the SodC monomer was identified (data not shown). The absence of immunoreactive bands when corresponding blots were incubated with an antibody directed against *H. ducreyi* 35000 outer membrane proteins indicated the lack of contaminating cell wall components (data not shown).

Identification of an iron ABC transporter periplasmic-binding protein by mass spectrometry

Six independent pairwise comparisons of 2-D gel protein profiles disclosed one spot consistently exhibiting increased expression under heme-limitation conditions compared to heme-replete conditions (ratio of 3.9, P value <0.03) (Fig. 1a, b). The peptide mass signature of the protein spot was determined by MALDI tandem mass spectrometry and the accuracy of the putative peptide identifications were evaluated by applying the Mascot scoring algorithm. The peptide mass fingerprint displayed significant amino acid homology (73%) to YfeA, a periplasmic-binding protein from *Y. pestis* involved in iron acquisition (Bearden and Perry 1998).

The molecular mass of the *H. ducreyi* 35000 putative iron periplasmic-binding protein calculated from 2-D gels was approximately 31 kDa, similar to

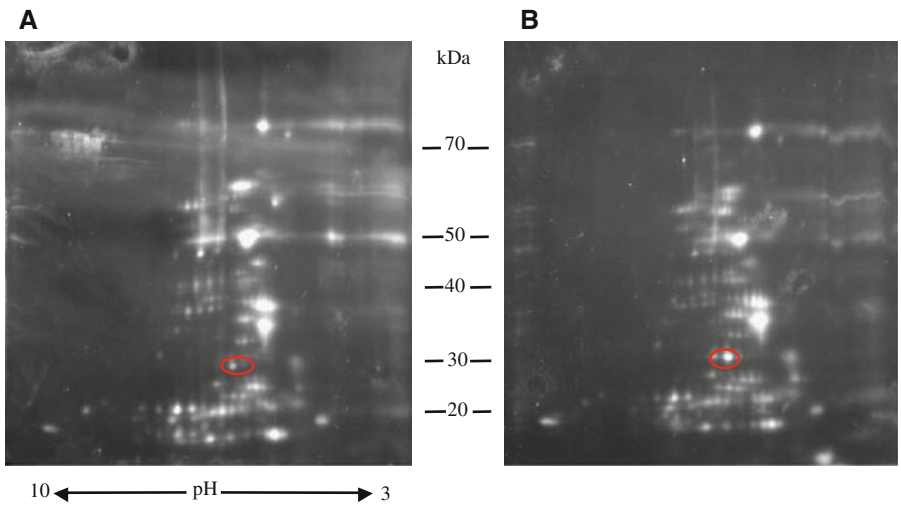

Fig. 1 Periplasmic proteome maps of *H. ducreyi* 35000 proteins isolated from periplasmic fractions of cells grown under heme rich (**a**) and heme deficient (**b**) conditions. Protein samples (10 μg) were separated by 2-D gel electrophoresis (pH range 3–10, molecular mass range 20–70 kDa), and the gels were stained with SYPRO Ruby followed by spot matching, spot abundance and statistical analysis. The circled spots identify a 31 kDa protein whose expression is increased under heme restriction

other characterized periplasmic-binding proteins (Zimmerman et al. 1989; Adhikari et al. 1995; Lewis et al. 1999). The *H. ducreyi* 35000 protein was designated hHbp (*H. ducreyi* heme periplasmic-binding protein). Querying the peptide mass fingerprint of hHbp against the sequenced genome of *H. ducreyi* 35000HP identified the responsible gene as HD1816. The 906 bp coding sequence predicted a protein consisting of 302 amino acids (aa) with a calculated MW of 33,240 and with a functional annotation as a solute-binding protein. Comparative PSI-BLAST (Position Specific Iterated BLAST) protein sequence alignments indicated that hHbp was homologous to several bacterial ABC periplasmic-binding proteins engaged in metal ion transport, specifically, 88% similar (residues 1–298) to the periplasmic adhesin component of a metal ion transport system in *Actinobacillus pleuropneumoniae*, 75% similar (residues 10–293) to an iron ABC transporter periplasmic binding protein in *H. influenzae* and 73% similar (residues 23–293) to *Y. pestis* YfeA (Fig. 3). An NCBI conserved domain search indicated that hHbp is a member of the TroA superfamily of transition metal solute-binding proteins (data not shown) (Desrosiers et al. 2007).

PSORT analysis identified a typical signal sequence, i.e., two positively charged residues followed by a hydrophobic stretch at the hHbp N-terminus (Gardy and Brinkman 2006). The predicted cleavage site of the signal sequence between residues 23 and 24 would result in a mature protein of ~31 kDa comprised of 278 aa that localized to the periplasmic space. The predicted molecular mass of the mature protein corresponded to the size of hHbp determined from 2-D gel analysis. PSORT results were confirmed experimentally with periplasmic, cytoplasmic, and outer membrane preparations from *H. ducreyi* 35000 run on 1-D SDS–PAGE followed by Western immunoblots probed with anti-hHbp polyclonal antiserum (data not shown). An immunoreactive band of ~31 kDa was present in all periplasmic samples with only weak immunoreactive bands present in cytoplasmic samples from *H. ducreyi* 35000 grown under heme-limiting conditions. Of note, the expression of hHbp was upregulated in periplasmic protein samples collected from *H. ducreyi* 35000grown under heme-limiting conditions compared to those grown under heme-replete conditions (data not shown).

Sequence analysis of the *hhbp* gene cluster

The hHbp gene was flanked by three open reading frames, corresponding to genes HD1814, HD1815 and HD1817, respectively, in the *H. ducreyi* 35000HP chromosome. The genes in the *hhbp* cluster are arranged contiguously, either overlapping or separated by gaps not exceeding five nucleotides. A potential promoter region was located immediately upstream of HD1814 preceded by a well conserved Shine-Dalgarno sequence appropriately spaced from the initiation codon. No associated Fur binding site, typical of iron-regulated genes, was detected. The translational start codon of hHbp and HD1817 overlap, indicating that these proteins are translationally coupled. An inverted repeat similar to Rho-independent transcriptional terminators was found 45 bp downstream of the HD1817 stop codon. The location of this stem loop structure downstream of the C-terminus of HD1817 suggested that this gene cluster is transcribed as a single transcriptional unit. The product of HD1814 revealed a protein containing six possible membrane-spanning segments, consistent with an inner membrane protein as predicted by the PHDhtm and PSORT algorithms (Gardy and Brinkman 2006). The amino acid sequence of HD1814 demonstrated significant homology to a putative membrane carrier or transport protein of unknown substrate specificity described in *Erwinia carotovora* (65%; residues 3–220), *S. flexneri* (61%; residues 2–220), *E. coli* (61%; residues 3–220), *H. somnus* (64%; residues 3–220), and *Pasteurella multocida* (63%; residues 1–221). Interestingly in these organisms, the gene immediately downstream of the homologous membrane carrier shows relatedness to the gamma subunit, designated as DsvC or DsrC (Pierik et al. 1992), of dissimilatory sulfite reductases. The enzyme exhibits a novel multimeric assembly comprising a reduced porphyrin covalently coupled to an iron-sulfur cluster and catalyzes the reduction of sulfite in sulfate reducing bacteria where sulfite acts as a terminal electron acceptor. An identical genetic configuration was observed in *H. ducreyi* as the adjacent HD1815 encodes a polypeptide with significant similarity to a protein found in *A. pleuropneumoniae* (80%) and in *H. influenzae* (67%) that is homologous to DsvC. The predicted HD1817 product encodes a protein exhibiting homology to the ATP-binding component of an ABC transporter system and contains the two Walker motifs (Walker A

13-GVNGGKST-22 and Walker B 163-DEPF-166) that have been identified in all nucleotide-binding proteins (Ames 1986; Higgins 1992; Doige and Ames 1993; Schneider and Hunke 1998). A third linker motif (139-LSGGQ-143) that typically precedes the Walker B motif is also present and is presumed essential for transmembrane solute transport (Stojiljkovic and Perkins-Balding 2002).

Expression and purification of recombinant hHbp

A protein of ~31 kDA, representing the mature form of hHbp following cleavage of the signal sequence, was seen in periplasmic fractions prepared from *E. coli* BL21 Star™ (DE3) cells harbouring the 6.7 kb pET151/D-TOPO®::*hhbp* construct (data not shown). Analysis of the remaining insoluble pellet by 1-D SDS–PAGE revealed a predominant protein band of ~37.2 kDA corresponding to the recombinant hHbp containing the hexahistidine tag inserted at the N-terminus (data not shown). These findings were confirmed by Western immunoblots of these cellular fractions probed with antibodies against the polyhistidyl hHbp fusion protein (data not shown). As the fusion tag was placed immediately upstream of the signal sequence, the recombinant hHbp was purified by Ni^{2+}-chelate chromatography from the cell lysates that remained after periplasmic extraction.

Exhaustive attempts to enzymatically cleave the N-terminal polyhistidine tag using the tobacco etch virus protease were unsuccessful, despite numerous trials in which the incubation time, incubation temperature, and ratio of rhHbp to enzyme were varied (data not shown). Therefore, the affinity-purified polyhistidyl recombinant hHbp fusion protein (His-hHbp) was used for antibody production and functional characterization.

Heme binding by His-hHbp

Two independent methods were used to demonstrate the heme binding properties of His-hHbp. First, increasing concentrations of purified His-hHbp were incubated with a fixed amount heme-agarose beads. The protein was eluted and samples were separated was by SDS–PAGE. The His-hHbp sedimented with the heme affinity matrix in a dose-dependent manner (Fig. 2). The absence of His-hHbp when the affinity resin alone was subjected to the binding protocol

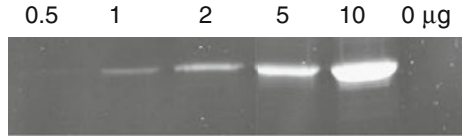

Fig. 2 Binding of His-hHbp to hemin-agarose. Increasing amounts of affinity-purified His-hHbp (0–10 µg) were incubated with 20 µl of hemin agarose (≥4 µmol/ml). Bound proteins were eluted by 2× sample buffer and samples were separated by 12% (wt./vol.) SDS–PAGE. The gel was stained with SYPRO Ruby

indicated the protein is not a degradation product from the agarose matrix. The interaction of His-hHbp with the immobilized heme was specific as retention of His-hHbp was inhibited in a concentration-dependent fashion by pre-incubating the protein with soluble heme (Fig. 3).

In the second approach, dose-dependent heme binding to the protein was detected by enhanced chemiluminescence (Fig. 4a, Panel ECL). In the corresponding Western blot, a mirror image of the ECL was obtained (Fig. 4a, Panel WB). As the anti-hHbp probe recognizes the unbound form of hHbp, the decrease in the hHbp immunoreactive band is a consequence of the binding of the protein to heme. This reciprocal relationship between free hHbp and heme is consistent with and mirrors the result obtained by ECL. These assays support a specific interaction between hHbp and heme. However, the precise kinetics of the interaction between hHbp and heme are difficult to establish, compromised by the recognized propensity of heme to form aggregates in aqueous solutions (Kuzelova et al. 1997).

As histidine residues serve as axial ligands in heme binding (Bracken et al. 1999), the possibility existed that the His-hHbp heme binding was mediated by the uncleaved hexahistidine fusion tag. To address this likelihood, the ability of a 31 kDa

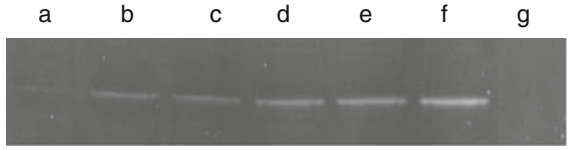

Fig. 3 Hemin competition binding assay. His-hHbp (10 µg) was pre-incubated with 0 (*lane f*), 300 µM (*lane e*), 400 µM (*lane d*), 600 µM (*lane c*), 800 µM (*lane b*) and 1 mM (*lane a*) hemin for 1 h followed by hemin agarose affinity-purification. *Lane g* represents a sample in which no His-hHbp was added. Samples were separated by 12% (wt./vol.) SDS–PAGE and the gel stained with SYPRO Ruby

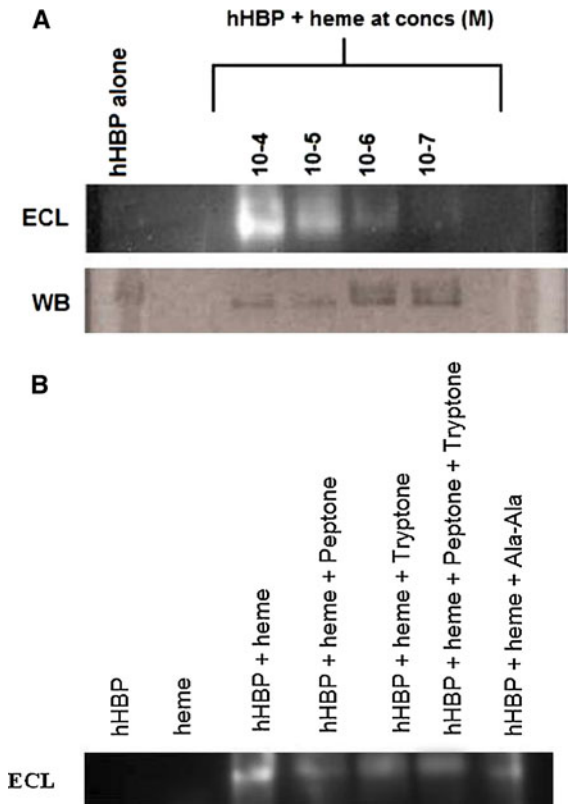

Fig. 4 Detection of heme binding by enhanced chemiluminescence. **a** Binding of heme to purified His-hHbp was detected by incubating hHbp with the indicated concentrations of heme. Following nondenaturing PAGE, the proteins were transferred onto a nitrocellulose membrane and heme complexed with protein bands was detected by enhanced chemiluminescence. In the corresponding Western immunoblot, the membrane was reacted with antibody raised against hHBP. **b** Peptide competition of heme binding was performed in a similar fashion by incubating 10 μg of purified hHbp with heme alone or with a mixture of heme, peptone and tryptone. ECL denotes enhanced chemiluminescence

recombinant outer membrane lipoprotein from *Leptospira*, rLipL32, possessing a polyhistidyl N-terminal tag, to bind heme was examined. The absence of detectable heme binding by enhanced chemiluminescence indicated that the specific interaction of heme with His-hHbp was not mediated by the histidine fusion tag (data not shown). This result is in accord with the demonstration that the presence or absence of an N-terminal 6-His epitope in SiaA, a periplasmic heme-binding protein in *Streptococcus pyogenes*, did not affect the NMR analysis of the heme methyl resonances of the protein, indicating that the N-terminal fusion did not interact with any heme molecules in solution and that the histidine tag did not alter the heme binding site (Sook et al. 2008).

Peptide inhibition of hHbp

As heme transport in *E. coli* involves the dipeptide permease *dppABCDF* (Létoffé et al. 2006), the ability of hHbp to recognize peptide substrates was assessed. In the ECL assay (Fig. 4b), mixtures of peptone and tryptone partially inhibited the interaction of heme with hHbp. This result indicates that hHbp displays a degenerate substrate specificity, reminiscent to that exhibited by the *E. coli* DppA periplasmic-binding protein (Létoffé et al. 2006). The physiological relevance of this modest peptide binding to hHbp is uncertain and awaits more detailed molecular and functional studies.

Competition binding assays

Protoporphyrin IX (PPIX) was more effective at displacing the binding of hHbp to hemin-agaose as concentrations of PPIX above 100 μM markedly reduced absorption of His-hHbp to the affinity gel (Fig. 5). Therefore, the interaction of hHbp with heme requires the tetrapyrrole moiety. The homology exhibited by hHbp to bacterial periplasmic-binding proteins functionally assigned as iron transporters suggested that hHbp may share this characteristic. The inability of either ferric nitrate or ferric chloride to competitively displace the binding of His-hHbp to the heme matrix excluded this possibility (data not shown).

Heme regulation of hHbp expression

Components of bacterial heme or iron acquisition pathways are typically expressed under conditions of

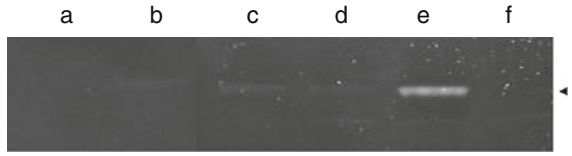

Fig. 5 Protoporphyrin IX competition binding assay. His-hHbp (10 μg) was pre-incubated with 0 (*lane e*), 50 μM (*lane d*), 100 μM (*lane c*), 300 μM (*lane b*), and 400 μM (*lane a*) protoporphyrin IX for 1 h followed by hemin agarose affinity-purification. *Lane f* represents a sample in which no His-hHbp was added. Samples were separated by 12% (wt./vol.) SDS–PAGE and the gel stained with SYPRO Ruby

heme or iron limitation (Stojiljkovic and Perkins-Balding 2002). To assess whether iron limitation influenced the expression of hHbp, 2-D gel profiles of periplasmic extracts from *H. ducreyi* 35000 cultures grown under heme replete conditions (77 μM) in the presence of increasing concentrations of desferrioxamine (10–200 μM) were compared. No increase in the measured spot intensity of hHbp was observed with increasing concentrations of the iron-chelating agent (data not shown). Furthermore, no increase in intensity of the hHBP immunoreactive band was observed in heme-restricted periplamic preparations containing 50 μM desferoxamine compared to extracts without the iron chelator (Fig. 6b). This concentration of desferrioxamine has been shown to impose iron limited growth in *Neisseria gonorrhoeae* (Lee and Schryvers 1988). These results indicated that iron restriction did not influence hHbp expression, suggesting that the uptake of heme iron might determine the size of the intracellular iron pool, and thus govern hHbp expression, as has previously been shown for the *H. ducreyi* outer membrane hemoglobin binding protein HgbA (Elkins 1995). Periplasmic extracts prepared from cells grown under low and high heme conditions were probed with polyclonal antiserum against hHbp in Western blot analysis. Expression of hHbp was increased under heme restriction (Fig. 6a). The absence of hHbp in the corresponding outer membrane and cytoplasmic fractions confirmed the periplasmic location of hHbp (Fig. 6a). These results indicate that the concentration of heme in the growth medium regulates the periplasmic expression of hHbp.

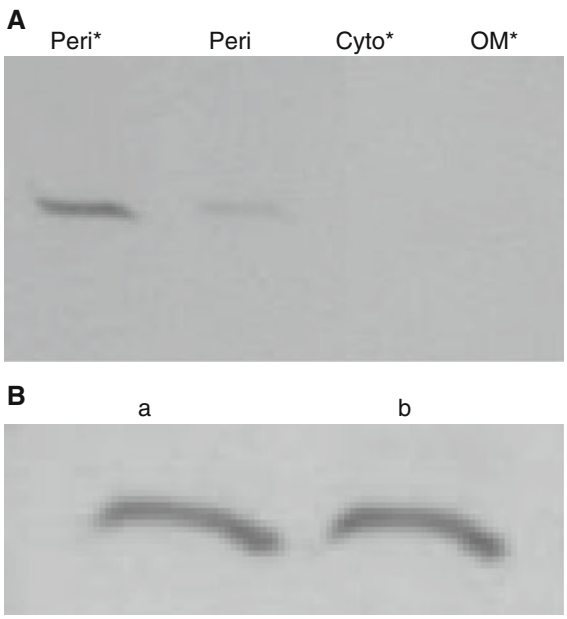

Fig. 6 Heme regulation of hHbp expression and hHbp periplasmic localization. **a** Western immunoblot analysis of *H. ducreyi* cell fractions prepared from cells grown under heme limiting (Peri*) and heme replete (Peri, Cyto, OM) conditions probed with anti-hHbp specific antiserum. Peri denotes the periplasmic extract, Cyto the cytoplasmic fraction and OM the outer membrane preparation. **b** Western immunoblot of *H. ducreyi* periplasmic extracts prepared from cultures grown under heme-restrictive conditions containing 0 μM (*lane a*) or 50 μM (*lane b*) of the iron chelator desferrioxamine

Genetic complementation

Although a nonpolar mutation in *hhbp* would provide direct genetic evidence of the role of this gene in heme acquisition in *H. ducreyi*, several attempts to construct such an isogenic mutant were unsuccessful. Therefore, the alternate approach of functional complementation of an *E. coli* heme uptake mutant was used.

Recombinant hHbp expressed in *E. coli* FB827 (pAM238-hasR) carrying the *dppA::Cm mppA::Km* double mutation localized to the periplasmic space as determined by Western blot analysis (data not shown). The plasmid construct pET151-hHbp bearing the wild-type *hhbp* under the control of the lac promoter rescued the ability of the *E. coli dppA::Cm mppA::Km* (pAM238-hasR) heme permease mutant to use heme as an iron source (Table 2). The amount of growth was similar to that observed in the *E. coli* heme uptake mutant FB827 *dppF::Km* (pAM238-hasR) transformed with the plasmid pTRC99-dppABCDF encoding the entire Dpp operon. The growth of this mutant has previously been shown to be equivalent to that of the parental isolate, *E. coli* FB827 (pAM238-hasR), in this assay (Létoffé et al. 2006). The failure of these constructs to restore growth on M63* Dip plates devoid of hemoglobin indicated that the growth promoted by both hHbp and the intact Dpp permease on M63* Dip media supplemented with hemoglobin resulted from facilitating heme–iron utilization, and not peptide transport. The complete absence of growth of the *dppA mppA* double mutant and of the same mutant transformed with the empty vector pET151 on the hemoglobin supplemented plates demonstrated that neither nonspecific diffusion of heme across the

Table 2 Complementation of the *E. coli* heme uptake mutant for hemoglobin use

Strains	Growth on M63* plates containing 2′2′dipyridyl and hemoglobin at concentrations of			
	0 μM	1 μM	5 μM	10 μM
E. coli FB827 *dppA*::Km *mppA*::Cm (pAM238-hasR)	–	–	–	–
E. coli FB827 *dppA*::Km *mppA*::Cm (pAM238-hasR) (pET151)	–	–	–	–
E. coli FB827 *dppF*::Km (pAM238-hasR) (pTRC99-dppABCDF)	–	+++	+++	+++
E. coli FB827 *dppA*::Km *mppA*::Cm (pAM238-hasR) (pET151-hHbp)	–	++	+++	+++

perilplasmic space, nor proteins expressed from the pET151 plasmid backbone, contributes to heme use, respectively.

Although *hhbp* was sufficient to fully complement heme utilization in the *E. coli* Dpp-heme permease mutant at hemoglobin concentrations in excess of 1 mM, the growth of this mutant carrying the *hhbp* gene was less confluent than that produced by the strain complemented with the whole Dpp operon when hemoglobin was supplied at a low concentration. Two explanations may have accounted for this observation. First, the low amount of hHbp expression in *E. coli* FB827 *dppA::Km mppA::Cm* (pAM238-HasR) (pET151-Hhbp) may have been insufficient to fully restore the heme competency of the complemented strain. A second more plausible reason for the difference in growth arises from the use of a heterologous protein to rescue the heme deficiency in the double mutant. A lack of stability of the *H. ducreyi* protein in the *E. coli* recipient or subtle structural differences between hHbp and the *E. coli* authentic periplasmic binding proteins DppA and MppA may have compromised the ability of hHbp to completely functionally complement the mutant strain.

In summary, these results indicate that hHbp is functional in *E. coli* and support the postulated role of this protein in heme acquisition in *H. ducreyi*.

Distribution of hHbp among clinical strains of *H. ducreyi* and *H. influenzae*

The *hhbp* locus was present and actively expressed in a limited panel of diverse *H. ducreyi* clinical isolates (data not shown). The identification of a similar ~31 kDa immunoreactive band in the four *H. influenzae* clinical strains suggested that this organism possesses an hHbp homologue (data not shown). The absence of the corresponding amplicon was not unexpected as the complementary oligonucleotides used to generate the amplified fragment shared incomplete identity to the *H. influenzae* target (Fig. 7).

Discussion

The identification of a putative *H. ducreyi* heme ABC transporter in this study was achieved by protein expression profiling of the *H. ducreyi* 35000 periplasmic proteome. Mapping of this subcellular compartment was enhanced by the use of the highly sensitive stain, SYPRO Ruby, a ruthenium fluorescent dye that exhibits a broad linear dynamic range (Pickford et al. 2004; Lopez et al. 2000). The success of this approach also derived from the selective targeting of the periplasmic-binding protein component of the transporter. The recognized inability of 2-D gels to adequately resolve membrane-associated proteins would have challenged attempts to identify the membrane embedded polypeptides of the transporter. The power of proteomics in uncovering bacterial transport systems engaged in heme acquisition is illustrated by a prior investigation into the global status of protein expression in response to exposure to excess environmental heme in *Staphylococcus aureus* (Friedman et al. 2006). A novel staphylococcal heme-regulated transport system, HrtAB, was disclosed using 2-D differential gel electrophoresis of cytoplasmic extracts.

The hHbp protein was identified in a previous proteomic study that constructed a preliminary *H. ducreyi* 35000HP whole cell reference map (Scheffler et al. 2003). Based on sequence similarity to an *H. influenzae* orthologue, the protein was deduced to be the periplasmic constituent of an iron-binding ATP transporter. Not surprisingly, this mistaken

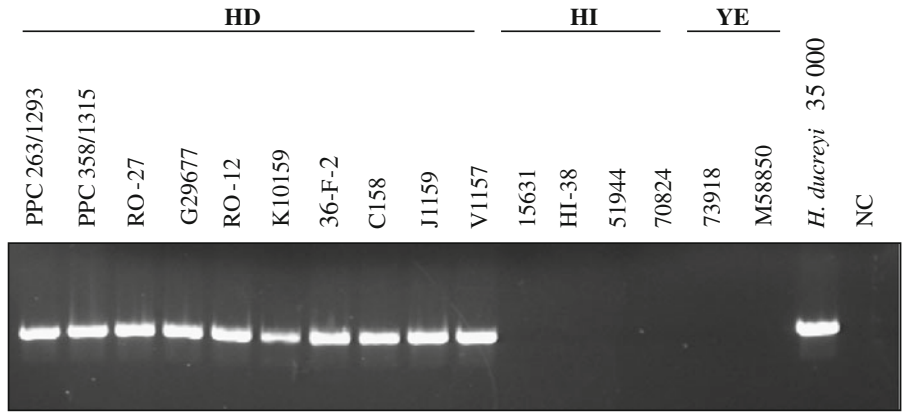

Fig. 7 Identification of *hhbp* among clinical isolates of *H. ducreyi*, *H. influenzae* and *Y. enterocolitica*. Genomic DNA from *H. ducreyi* (HD), and *H. influenzae* (HI), and *Y. enterocolitica* (YE) served as template for PCR amplification reactions using *hhbp* specific primers. Amplicons were separated on a 1% (vol./vol.) agarose gel and the gel was stained with ethidium bromide. NC is a sample containing no DNA template

assignment was reiterated in our study as the closest matches to hHbp in the protein database directed search were to periplasmic-binding proteins involved in metal ion or iron transport in several Gram negative bacteria, including *H. influenzae*. Such a result reinforces the requirement for experimental verification of protein function, as function prediction by homology is prone to systematic annotation errors (Devos and Valencia 2001; Koski and Golding 2001).

Several striking features emerge from an analysis of the *H. ducreyi hhbp* locus. First, the gene cluster does not conform to the prototypical genetic organization of bacterial ABC transporters in which the gene encoding the periplasmic-binding protein is promoter proximal. In contrast, the hHbp ORF is the second gene in the cluster. Gene order forms the basis for translational coupling, whereby the rate of translation of a gene on a polycistronic transcript is dependent on the translation of the preceding gene. This phenomenon has been invoked to account for the coordinate expression of genes in several *E. coli* operon systems (McCarthy 1990; Schumperli et al. 1982). Therefore, one potential consequence of this altered genetic configuration is an effect in the differential expression of individual genes in the *hhbp* operon. A cardinal feature of bacterial binding-protein dependent importers is the preferential production of the periplasmic-binding protein constituent (Torres and Payne 1997). This characteristic is functionally relevant as the efficiency of the transport process is critically dependent upon the preservation of such a stoichiometry (Mademidis and Koster 1998).

A second peculiarity of the *hhbp* gene cluster is the presence of an ORF (HD1815) displaying amino acid homology to *dsvC*, a gene that encodes the γ subunit of dissimilatory sulfite reductase in *Desulphorvibrio vulgaris* (Pierik et al. 1992). This heme-containing enzyme catalyzes the reduction of sulfite in sulfate-reducing bacteria, in which sulfite serves as a terminal electron acceptor. Homologues of the γ-encoded polypeptide have been identified in non-sulfate-reducing bacteria, such as *E. coli* and *H. influenzae* (Molitor et al. 1998), and have been proposed to be involved in the assembly, folding and stabilization of siroheme proteins in these organisms (Molitor et al. 1998). The DsvC protein may serve an analogous role in *H. ducreyi*, which also lacks the sulfate reductase enzyme complex, by facilitating the interaction between heme and its cognate ABC transporter. The organization of the *hhbp* locus as a likely single transcriptional unit, resulting in the co-ordinate expression of the *dscC* gene with those for the permease, periplasmic-binding protein and ATPase-binding components of the transporter, supports this putative association.

Similar to the expression of TdhA and HgbA, heme deprivation appears to trigger the synthesis of hHbp. The simultaneous activation of the genes encoding the outer membrane receptors of a substrate acquisition network in concert with its dedicated ABC transporter is a feature common to high affinity transport systems

(Ames 1986; Doige and Ames 1993). Given the pronounced heme requirements exhibited by *H. ducreyi* (Hammond et al. 1978; Albritton et al. 1981), it is likely that the organism employs multiple parallel paths to funnel periplasmic deposited heme into the cytoplasm. Consistent with this proposal is the observation that an ortholog of *dppBCDF* (HD0312-0316) is present in *H. ducreyi*. The *dppBCDF* homolog in another heme dependent bacterium, *H. influenzae*, has been implicated in heme acquisition (Morton et al. 2009). Furthermore, the *H. influenzae* DppA homolog shares significant sequence similarity to HbpA, a periplasmic located lipoprotein that is involved in heme utilization in this organism (Morton et al. 2005). A version of HbpA exists in *H. ducreyi* (Hanson et al. 1992) and likely represents the functional equivalent of the *E. coli* DppA. Such a functional redundancy, mirroring that displayed at the cell surface, would provide a safeguard to the supply of this critical nutrient to this heme obligate bacterium.

An understanding of the precise role of both the *hhbp* gene cluster and the heme periplasmic-binding protein hHbp in the delivery of heme into the interior of *H. ducreyi* awaits the construction of genetically defined transport mutants.

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