Iron sources for *Haemophilus ducreyi*

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Summary. The ability of various haem- and non-haem-iron-containing compounds to support the growth of iron-limited cultures of *Haemophilus ducreyi* was assessed in a plate bioassay. Only haemin or the haem-containing proteins, bovine haemoglobin, human haemoglobin and bovine catalase, but not equine cytochrome C₁₁₁, were capable of serving as the sole exogenous iron source. Complexes of haptoglobin-haemoglobin and haem-serum albumin retained the ability to function as iron substrates. In contrast, no growth was observed with FeCl₃, human lactoferrin and human transferrin. Siderophore production was not detected with a universal chemical assay. Outer-membrane-protein profiles derived from iron-starved cultures revealed four iron-regulated polypeptides of 65, 50, 45·5 and 40·5 Kda. These results indicate that haem can supply the requisite iron for growth of *H. ducreyi*.

Introduction

The requirement for iron is a ubiquitous phenomenon of living cells.^{1,2} A plethora of experimental evidence supports the contention that the ability of a bacterial pathogen to acquire iron governs its pathogenic potential and represents an essential component of its virulence repertoire.^{1,2} However, the human body affords a hostile, iron-restrictive environment to a bacterial invader. Iron is sequestered in haem-containing proteins such as haemoglobin or myoglobin, and in ferritin or complexed to the iron-binding glycoproteins, lactoferrin (Lf) and transferrin (Tf), which in aggregate limits the available free iron to levels insufficient to support microbial growth.^{1,2}

In response, bacteria have developed sophisticated high-affinity systems to scavenge this essential element from their external environment. One such method involves the elaboration of low molecular weight iron-chelators, called siderophores, that solubilise and bind iron prior to its internalisation.^{1–3}

Recently, a receptor-mediated mechanism for the uptake of iron from Lf and Tf has been described in *Haemophilus influenzae*^{4,5} and in the pathogenic *Neisseria* spp.^{6,7} The Lf receptors (LfR), and to a lesser extent the Tf receptors (TfR), of these organisms exhibit similarities in their molecular weights and in their ligand and functional specificities, implying an interspecies structural and functional conservation of these receptors.

H. ducreyi, a fastidious gram-negative coccobacillus causes chancroid, a sexually transmitted genital ulcer disease.⁸ Although this organism also possesses an obligate requirement for haem,^{9,10} nothing is known about its iron acquisition mechanisms.

The recent epidemiological demonstration that genital ulcer disease is an independent risk factor for the acquisition of HIV¹¹ coupled with the re-emergence of chancroid as a significant sexually transmitted disease (STD) in North America,¹² has spurred renewed interest in this pathogen. However, studies on the metabolism of *H. ducreyi* have been hampered, in part, by the dearth of suitable in-vitro broth culture media.^{9,10,13}

Therefore, this investigation was conducted initially to develop a plate bioassay to circumvent the impediment imposed by the lack of broth media to support the growth of *H. ducreyi*. The development of this bioassay system permitted the identification of physiological iron-containing compounds that might be anticipated to furnish the requisite iron for growth in *H. ducreyi*, in order to provide an initial understanding of the mechanisms for iron uptake in this organism and to compare them with the mechanisms in *H. influenzae*.

Materials and methods

Bacterial strains

H. ducreyi strains, kindly provided by R. Brunham (University of Manitoba, Winnipeg, Canada) (table I), were maintained in skimmed-milk stock cultures at –70°C. *N. meningitidis* BI6B6 was a Group B:2a serotyping strain.⁷ *Streptococcus pneumoniae* ATCC27336 and *S. pyogenes* ATCC21547 were used as positive controls in the haemolysin assay. *Pseudomonas aeruginosa* PAO1¹⁴ was provided by L. E. Bryan (University of Calgary, Canada). When needed, strains were cultured on chocolate agar (CA)—GC Medium Base (Difco Laboratories, Detroit, MI, USA)

Table I. *H. ducreyi* strains

Strain no.	Source	β -Lactamase production
HD1	Sweden	—
CH28	Thailand	+
35000	Winnipeg, Manitoba, Canada	—
CIP542	Institut Pasteur, Paris, France	—
V1157	Seattle, Washington	+
BG411	Nairobi, Kenya	+
36-F-2	Paris, France	—
E1673	Sweden	—

supplemented with bovine haemoglobin (Difco) 1% and CVA Enrichment (Gibco Canada Inc., Burlington, ON, Canada) 1%—incubated at 35°C in air + CO₂ 5% with high humidity.

Growth on CA was identified as *H. ducreyi* by the non-mucoid, yellow-grey appearance and the cohesive nature of the colonies which enabled them to be pushed intact across the agar surface. Biochemically, they were oxidase-positive and catalase-, urease-, and ornithine decarboxylase-negative; they did not produce indole or H₂S, and did not ferment glucose, sucrose, lactose, mannitol or xylose. Microscopy showed that all organisms were small, pleomorphic, gram-negative rods.

Iron-containing compounds

Iron-containing compounds were purchased from Sigma Chemical Co., St Louis, MO, USA. The procedures used to prepare the iron-loaded human lactoferrin (hLf) and human transferrin (hTf) were described previously.⁷ Bovine haemin (bHm), bovine haemoglobin (bHb), human haemoglobin A (hHb), equine cytochrome C₁₁₁ and bovine catalase were prepared fresh daily. With the exception of bovine haemin, which was solubilised initially in 0.1 N NaOH, the haem proteins were dissolved in deionised water and sterilised by membrane filtration immediately before use.

Reagents for siderophore detection

Chrome azurol S (CAS) and hexadecyltrimethylammonium bromide (HDTMA) were purchased from Fluka Chemical Corp., Ronkonkoma, NY, USA. Pipes (free acid), the salts comprising the MM9 growth medium¹⁵ and FeCl₃·6H₂O were obtained from Sigma.

Media

The semi-defined agar medium (DM) used comprised GC medium base (Difco) with glucose (Sigma) 0.1%, L-glutamine (Sigma) 0.01% and L-cysteine HCl (Sigma) 0.025%.

Serum proteins

Human serum albumin (hSA) and human haptoglobin (hHp), which comprised a mixture of 1:1, 2:1 and 2:2 electrophoretic variants,¹⁶ were obtained from Sigma. Complexes of haem with hSA and of hHb with hHp were prepared as previously described.¹⁷ The carrier proteins were saturated at 50% capacity, assuming a 1:1 stoichiometry of binding of haem to hSA and of hHb to Hp.¹⁸

Isolation and characterisation of outer-membrane proteins (OMPs)

OMPs were isolated by the method of Hancock and Nikaido,¹⁹ as modified by Godfrey *et al.*,²⁰ from organisms grown under the following conditions: iron replete [CA supplemented with CVA 1%], haemin-rich [DM containing 200 μ M ethylenediamine Di-(*O*-hydroxyphenylacetic acid) (EDDA) and 200 μ M bovine haemin], iron- and haemin-limiting [DM containing 38 μ M bovine haemin and 200 μ M EDDA] and haemin-restrictive [DM with the addition of 38 μ M bovine haemin]. Protein concentration was determined by the method of Lowry²¹ with bovine serum albumin as a standard. The proteins were separated by discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 1.5-mm thick slab gels with stacking 5% w/v and gradient acrylamide (BioRad Laboratories, Richmond, CA, USA) 8–12% separating gels with bisacrylamide (BioRad) 0.8%, and the buffer system of Laemmli.²² Gels were loaded with 50 μ g of protein per lane. The resultant gels were stained with silver.²³ Molecular weights were determined with known proteins as standards.

Hb-Hp complexes were analysed by non-denaturing PAGE, a protocol in which SDS was eliminated from the sample gel and running buffers and the sample was not subjected to thermal denaturation before electrophoresis. After reduction of the gel in methanol 50%,²⁴ haemoglobin and Hb-Hp complexes were stained for peroxidase activity by the benzidine stain described by Moore.²⁵

Plate assays

Because *H. ducreyi* exhibits intercellular adherence, uniform suspensions of this organism are difficult to achieve. This intrinsic character precluded the adaptation of traditional plate assays, in which organisms are incorporated in molten agar, to evaluate the availability of iron from haem and non-haem proteins. Therefore, to determine the minimum haemin requirement necessary to support growth, organisms derived from a 24-h culture on CA were suspended in 50 mM sodium phosphate buffer, pH 7.0. The optical density was adjusted to a 0.5 McFarland standard (approximately 10⁸ cfu/ml). By means of a Steer's replicator, which delivered approximately 10⁵ cfu, organisms

were inoculated on to DM containing serial two-fold dilutions of bovine haemin. Plates were incubated at 35°C in air + CO₂ 5%. The presence or absence of growth was assessed with a hand lens after incubation for 24, 48 and 72 h. Only plates on which there was visible growth yielded viable organisms on subculture to CA.

A similar procedure was used to assess the ability of the various iron-containing compounds to support growth. Organisms were sequentially passaged once on CA, DM plus 38 µM bovine haemin, and DM plus 38 µM bovine haemin and 200 µM EDDA. The incorporation of EDDA, a potent iron chelator,⁷ into the DM complexed any available free iron in the medium. A preliminary series of titrations indicated that this concentration of EDDA was sufficient to impose iron limitation, as reflected in the Tf and Lf receptor expression of *N. meningitidis* strain B16B6 (see below). Organisms grown under these iron- and haemin-restricted conditions were then inoculated with a Steer's replicator on to similar EDDA-containing plates in which the following iron-containing compounds were individually substituted *in lieu* of bovine haemin: 9.5 µM bHb, 50 µM FeCl₃, 250 µM FeCl₃, 7.5 µM hTf, 1 µM hLf, 9.5 µM catalase, and 38 µM cytochrome C₁₁₁. Because of the haem dependency of *H. ducreyi*, plates containing the non-haem iron compounds, FeCl₃, hTf and hLf, were supplemented with the haem precursor, protoporphyrin IX (PPIX), at a concentration of 38 µM. Human Hb was added at a final concentration of 38 µM Fe, either free or complexed to hHp. Human haemin bound to hSA was added at a final concentration of 38 µM Fe. All assays were performed in triplicate.

Detection of siderophore production

Because the inability of *H. ducreyi* to grow in liquid media precluded the application of conventional bioassays for the detection of siderophores, the chemical method of Schwyn and Neilands¹⁵ which could be readily adapted for use on agar plates, was used. A further advantage inherent in this method was its universality, which facilitated the detection of structurally novel siderophores that possess functional groups other than hydroxamates or catechols. Organisms rendered iron- and haemin-deficient by sequential passage, as previously described, were inoculated by a Steer's replicator on to DM containing 38 µM bHm and the plates were incubated at 35°C in air + CO₂ 5%. Following the appearance of visible growth after incubation for 36–48 h, the plates were overlaid with 20 ml of CAS agar.¹⁵ Siderophore excretion, as indicated by the presence of orange haloes around the colonies, was assessed with reference to the indicator strains, *P. aeruginosa* PAO1 and *N. meningitidis* B16B6, after incubation for 24, 48 and 72 h at 35°C. All tests were performed in triplicate. This overlay modification was necessary because toxicity of the detergent

component, HDTMA, for *H. ducreyi* (data not shown) prevented the use of the original method of direct plating on to CAS agar.

Lf and Tf receptor dot-binding assay

Organisms and OMP preparations derived from whole cells grown on DM with 38 µM haemin and 200 µM EDDA were probed for the presence of Lf and Tf receptor expression as described previously.⁶ *N. meningitidis* B16B6 was tested as a positive control.

Haemolysin assay

To detect the presence of haemolysins, organisms were streaked on to and "stabbed" into modified blood-agar plates consisting of 10 ml of DM with 38 µM bovine haemin overlaid with a second 10 ml of DM containing bovine haemin and either defibrinated sRBC 5% or defibrinated hRBC 5%. Concurrent plates were rendered iron-limiting by the addition of 200 µM EDDA. Cultures were incubated at 35°C in air + CO₂ 5% or anaerobically at 35°C in an Anaerocult (BDH Chemicals Canada Ltd, Edmonton, AB, Canada) system. Haemolytic activity was assessed after 24, 48, 72 and 96 h.

Results

Haem requirement of *H. ducreyi*

All the *H. ducreyi* strains exhibited an obligate requirement for haemin; a minimum concentration of 38 µM (25 µg/ml) was necessary to initiate growth. The substitution of protoporphyrin IX, the immediate precursor of haem in its biosynthetic pathway, did not support growth.

Plate bioassays to determine the use of iron-containing compounds

Molar equivalents of haem as bHm, bHb, hHb, and catalase supported the growth of all the *H. ducreyi* isolates (table II) with growth detected after incubation for 48 h, but cytochrome C₁₁₁ did not support growth. Although no qualitative growth differences between cultures on media containing the various defined haem proteins were observed, there was some interstrain heterogeneity in the ability of these compounds to act as the sole iron source. In contrast, no strains were able to obtain the iron required for growth from hTf (fig. 1) or hLf. All the *H. ducreyi* strains assimilated FeCl₃ poorly; little or no growth was present even after prolonged incubation (> 72 h) (table II). *N. meningitidis* B16B6 was capable of utilising all of the compounds as the sole exogenous source of iron.

Table II. Acquisition of haem from various haem-containing proteins

Strain no.	Haem acquired from									
	FeCl ₃	hLf	hTf	hHb	hHp	cytochrome C ₁₁₁	catalase	bhm	hHb-hHp	hm-hSA
<i>N. meningitidis</i> B16B6	+	+	+	+	+	+	+	+	+	+
<i>H. ducreyi</i>										
HD1	—	—	—	+	+	—	+	+	+	+
CH28	—	—	—	+	+	—	+	+	+	+
35000	—	—	—	+	+	—	+	+	+	+
CIP542	—	—	—	+	+	—	+	+	+	+
V1157	—	—	—	+	+	—	+	+	+	+
BG411	—	—	—	+	+	—	+	+	+	+
36-F-2	—	—	—	+	+	—	+	+	+	+
E1673	—	—	—	+	+	—	+	+	+	+

Fig. 1. Growth response of *H. ducreyi* 35000 and *N. meningitidis* B16B6 to hHb and hTf after incubation for 48 h on iron-limited DM containing (A) 38 μ M hHb and (B) 7.5 μ M of 100% saturated hTf as the sole iron source. Row 1, Indian ink reference point; 2, *N. meningitidis* B16B6; 3, *H. ducreyi* 35000.

Effect of serum carrier proteins on growth

Haem bound to hSA, and hHb bound to hHp, remained suitable iron substrates because the haem was readily available for growth (table II). Non-denaturing PAGE of apo-hHp, free hHb and mixtures of the two confirmed that the hHp preparations were capable of binding hHb (data not shown). The addition of hHb to hHp resulted in a retardation of the electrophoretic mobility of both hHb and hHp, consistent with hHp-hHb complex formation.

Analysis of siderophore production

Siderophore production was not detected in *H. ducreyi* by the modified CAS agar assay. Appropriate reactions were obtained with the positive control strain, *P. aeruginosa* PAO1, which elaborates hydroxamate (pyoverdine)²⁶ and phenolic (pyochelin)²⁷ siderophores, and with the negative control, *N. meningitidis* B16B6, which does not produce any siderophores.²⁸

Expression of Lf and Tf receptors

The results from the Lf and Tf receptor solid-phase dot-binding assays complemented the plate assays by demonstrating the functional inability of *H. ducreyi* to

use hLf and hTf as the sole exogenous iron source. No receptor activity was detected in whole-cell and OMP preparations of *H. ducreyi* under iron-limiting conditions that permitted the expression of such receptor activity in *N. meningitidis* B16B6.

OMP profiles

The OMP profiles of *H. ducreyi* 35000 and *N. meningitidis* B16B6 grown under iron-replete and iron-limited conditions are shown in fig. 2. Comparison of

Fig. 2. Silver stained OMP profiles from gradient (8–12%) SDS-PAGE of *N. meningitidis* B16B6 (lanes a–d) and *H. ducreyi* 35000 (e–h) grown under iron-limited and iron-replete conditions. Mol.-wt markers are shown. Lanes a and e, cells grown on CA; b and f, cells grown under haemin-limiting conditions (DM with 38 μ M haemin); c and g, cells grown under haemin- and iron-deficient conditions (DM with 38 μ M haemin plus 200 μ M EDDA); d and h, cells grown under haemin-rich conditions (DM with 200 μ M haemin plus 200 μ M EDDA). Iron-regulated OMPs are indicated by arrow-heads (<).

N. meningitidis B16B6 samples from cells grown under iron-rich (lanes a and d) and iron-limited (lane c) conditions clearly shows three previously described⁷ iron-regulated OMPs of 71, 100 and 69 Kda. The last two polypeptides correspond to the reported M_r of the meningococcal Tf receptor.⁷ An identical OMP pattern was observed in membranes prepared conventionally from cultures grown in BHI broth rendered iron-limited. Under similar conditions, four OMPs of 65, 50, 45.5 and 40.5 Kda were detected in preparations from *H. ducreyi* 35000. The appearance or enhanced expression of these proteins only under iron- and haem-deficient conditions in which haemin served as the sole source of required iron (fig. 2, lane g), but not under haem-restrictive conditions (fig. 2, lane f), and their absence from OMP preparations from cells grown in haem-rich conditions (fig. 2, lanes e and h) indicate that they represent iron-repressible OMPs. In contrast to *N. meningitidis* B16B6, no iron-regulated polypeptides of 69 or 100 Kda were present in OMP preparations from *H. ducreyi* 35000.

Haemolysin assay

All *H. ducreyi* strains produced β -haemolysis. Although no qualitative differences were noted between stab and surface cultures or between iron-replete and iron-limited cultures, there were minor interstrain differences in the degree of haemolysis. The putative haemolysin did not demonstrate species specificity; equivalent haemolysis was produced with sRBC and hRBC. There was no haemolysis under anaerobic conditions.

Discussion

The natural sources of iron for *H. ducreyi* are unknown. This study indicates that haem can supply the necessary iron to support the growth of *H. ducreyi*. The pronounced partiality for haem is further exemplified by the ability of serum carrier protein complexes of haem or haemoglobin to provide the requisite iron for growth. Such a characteristic is shared by *H. influenzae*.²⁹ In contrast, neither *N. gonorrhoeae* nor *N. meningitidis* are capable of extracting haem iron bound to hSA.³⁰

However, because of its intrinsic limitation in providing only qualitative data, the plate bioassay method was not suitable for distinguishing minimal quantitative differences in growth stimulation amongst the various haem-containing compounds. Such differences in growth kinetics would be valuable in the optimal formulation of media constituents for primary isolation of *H. ducreyi*.

Most haem proteins reside intracellularly. Although their physiological relevance remains to be determined, the production of a haemolysin, as demonstrated in this study and previously,³¹ and exotoxins³² would facilitate access to these iron compounds.

In contrast to the other haem proteins, the inability of cytochrome C_{111} to promote growth could be explained by the relative inaccessibility of its porphyrin ring. This would be expected because of the covalent binding of the haem prosthetic group to its constituent protein coupled with the reduced surface exposure of haem in cytochrome C_{111} .^{33,34}

The inability of the non-haem compounds to serve as iron sources was not unexpected. Because *H. ducreyi* is haem-dependent, the observed limitation of growth in the presence of non-haem iron compounds may be a reflection of haem restriction rather than of iron deprivation. Since the specific defect(s) in the haem biosynthetic pathway of *H. ducreyi* is not known,^{9,10} the provision of the immediate haem precursor protoporphyrin IX (PPIX), in the presence or absence of a specific non-haem iron source, was intended to surmount this problem of interpretation. However, the failure of the apoprotein form of haem, PPIX, alone to abrogate the haem dependency of *H. ducreyi*, indicates that the organism cannot assimilate PPIX or lacks the enzyme ferrocatalase that catalyses the insertion of iron into the porphyrin ring, or lacks both abilities. Thus, although conventional iron-uptake and growth studies would discriminate between haem and iron deprivation and would enable determination of whether non-haem iron compounds can serve as iron sources, the inability to produce undispersed liquid cultures of *H. ducreyi*¹³ precludes the use of these techniques to address these critical issues. However, it is intriguing to note that the apparent absence of a functional Tf and Lf receptor in *H. ducreyi* may explain the inability of these glycoproteins to serve as iron sources.

In contrast, in *H. influenzae*, another obligately haem-requiring bacterium,³⁵ haem is only one of several forms of iron that satisfies this organism's iron requirements.²⁹ Being nutritionally more versatile, *H. influenzae* can extract iron from diverse sources, including Tf.^{29,36}

The absence of siderophore production in *H. ducreyi* prompts several speculations. Firstly, the observation that haem is actively transported as an intact molecule in *H. influenzae*³⁷ indicates that the acquisition of iron from haem-containing proteins by *H. ducreyi* may involve interaction with a surface exposed haem-specific receptor. The expression of several iron-regulated OMPs by *H. ducreyi* is consistent with this proposal; iron-repressible OMPs in other gram-negative bacteria function as receptors involved in iron uptake.² This contention is supported by observations in *H. influenzae* and *N. gonorrhoeae*, two other specific bacterial pathogens of man. In *H. influenzae*, similar haem-repressible OMPs have been demonstrated which have been suggested as receptors in the haem acquisition pathway.^{17,37} The gonococcus expresses a differential subset of iron regulated OMPs depending upon the specific iron source used.³⁸

Lastly, the exclusion of a siderophore component in the uptake pathway, coupled with the apparent

absence of functional receptors for Tf and Lf, indicates that *H. ducreyi* may have developed a unique haem-restrictive iron acquisition pathway.

More extensive biochemical and genetic studies are required to corroborate these conjectures. However, this study indicates that *H. ducreyi* has developed iron acquisition mechanisms that may be unique amongst *Haemophilus* spp. and it may serve as the prototype

organism in the understanding of haem and porphyrin physiology.

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