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 C1, 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. 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. 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.

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.

Recently, a receptor-mediated mechanism for the uptake of iron from Lf and Tf has been described in *Haemophilus influenzae* and in the pathogenic *Neisseria* spp. 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. Although this organism also possesses an obligate requirement for haem, 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 HIV coupled with the re-emergence of chancroid as a significant sexually transmitted disease (STD) in North America 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.

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* B16B6 was a Group B:2a serotyping strain. *Streptococcus pneumoniae* ATCC27336 and *S. pyogenes* ATCC21547 were used as positive controls in the haemolysin assay. *Pseudomonas aeruginosa* PAO1 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)
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 haemoglobin (bHm), bovine haemoglobin (bHb), human haemoglobin A (hHb), bovine haemin, which was solubilised initially in 0.1 M NaOH, and bovine catalase were prepared fresh daily. With the exception of bovine haemin, which was solubilised initially in 0.1 M NaOH, the haem proteins were dissolved in deionised water 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

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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%.
were inoculated on to DM containing serial two-fold dilutions of bovine haematin. 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 haematin, and DM plus 38 μM bovine haematin and 200 μM EDDA. The incorporation of EDDA, a potent iron chelator, 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: 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 haematin 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 Neilands 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 with the haem precursor, protoporphyrin IX (PPIX), at a concentration of 38 μM. Following the appearance of visible growth after incubation for 36–48 h, the plates were overlaid with 20 ml of CAS agar. 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 haematin and 200 μM EDDA were probed for the presence of Lf and Tf receptor expression as described previously. 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 haematin overlaid with a second 10 ml of DM containing bovine haematin 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 haem; 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 I) with growth detected after incubation for 48 h, but cytochrome C₁₁, 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. I) 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

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<th>Strain no.</th>
<th>Haem acquired from</th>
<th>FeCl₃</th>
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<th>hHb</th>
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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 μM hHb and (B) 7.5 μM 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 (pyoverdin)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

![OMP profiles](image-url)
N. meningitidis B16B6 samples from cells grown under iron-rich (lanes a and d) and iron-limited (lane c) conditions clearly shows three previously described \(^7\) 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 \(\beta\)-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 to facilitate access to these iron compounds.

In contrast to the other haem proteins, the inability of cytochrome C\(_{11}\), 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\(_{11}\). \(^33,34\)

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, \(^5,10\) 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. \(^37,38\) 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 andLf, 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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References