Role of the *Haemophilus ducreyi* Ton System in Internalization of Heme from Hemoglobin

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By cloning into *Escherichia coli* and construction of isogenic mutants of *Haemophilus ducreyi*, we showed that the hemoglobin receptor (HgbA) is TonB dependent. An *E. coli* hemA tonB mutant expressing *H. ducreyi* hgbA grew on low levels of hemoglobin as a source of heme only when an intact *H. ducreyi* Ton system plasmid was present. In contrast, growth on heme by the *E. coli* hemA tonB mutant expressing hgbA was observed only at high concentrations of heme, was TonB independent, and demonstrated that *H. ducreyi* HgbA was not sufficient to function as a typical TonB-dependent heme receptor in *E. coli*. Allelic replacement of the wild-type *H. ducreyi* exbB, exbD, and tonB loci with the exbB, exbD, and tonB deletion resulted in an *H. ducreyi* isogenic mutant unable to utilize hemoglobin but able to utilize hemin at the same levels as the parent strain to fulfill its heme requirement. This finding confirms the TonB dependence of HgbA-mediated hemoglobin utilization and suggests that uptake of hemin in *H. ducreyi* is TonB independent. Additionally, the *H. ducreyi* Ton system mutant synthesized increased amounts of HgbA and other heme-regulated outer membrane proteins, consistent with derepression of these proteins due to lower intracellular heme and/or iron concentrations in the mutant. Sequencing of the Ton system genes revealed that the arrangement of the genes was exbB exbD tonB.

The proximity and structure of these genes suggested that they are transcribed as an operon. This arrangement, as well as the DNA and deduced amino acid sequences of these *H. ducreyi* genes, was most similar to those from other Pasteurellae.
of outer membrane receptors (23, 56). An hgbA isogenic mutant of *H. ducreyi* cannot bind or utilize hemoglobin as a source of heme but can utilize free heme, implying that the utilization of hemin does not require HgbA. It has been shown by Stevens et al. (56) that an isogenic mutant of hgbA (hap) expressed reduced virulence in an animal model of *H. ducreyi* infection.

In the course of these functional studies, we found that an *E. coli* hema mutant expressing hgbA binds hemoglobin yet does not grow on hemoglobin as a porphyrin source (23). This result suggested that additional components are necessary for utilization of hemoglobin after the binding step. The objective of the present study was to identify the additional components necessary for removal of heme from hemoglobin and for the transport of heme across the outer membrane.

### MATERIALS AND METHODS

#### Strains and media

Bacterial strains used in this study are described in Table 1.

<table>
<thead>
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<th>Strain or plasmid</th>
<th>Relevant genotype and/or phenotype</th>
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<td>pUNCH 1210</td>
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<tr>
<td></td>
<td>insert in EcoRI site, Kan*</td>
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</table>

#### DNA manipulations

Standard recombinant DNA methods were used as described in reference 52 or following manufacturers’ instructions.

The 8.5-kb EcoRI fragment of pUNCH 579 containing the entire hgbA gene and promoter was ligated into the EcoRI site of pACYC184 (15) to construct pUNCH 556. Expression of hgbA in *E. coli* IR754(pUNCH 556) was confirmed by Western blotting as previously described (22).

For construction of a library from strain 35000, chromosomal DNA was partially restricted with Sau3AI under limiting enzyme conditions, and 5- to 12-kb fragments were isolated from a preparative gel. These fragments were ligated into the NotI site of purified plasmid SKC (see Table 1). To avoid restriction fragment overlap, the subsequent host strains, *E. coli* DH5αMC were transformed with the ligation mixture, the resulting ampicillin-resistant transformants were pooled, and plasmid DNA was isolated by using the Magic Miniprep procedure (Promega). To clone the Ton system, this plasmid pool was electroporated into *E. coli* IR754(pUNCH 556) and selection was performed on LB agar containing hemoglobin (100 μg/ml), ampicillin (100 μg/ml), and tetracycline (10 μg/ml).

#### DNA sequencing and analysis

DNA sequence analysis was performed at the University of North Carolina at Chapel Hill Automated Sequencing Facility using Taq terminator chemistry. Both strands were completely sequenced and assembled by using AssemblyLIGN software (IBI). Amino acid alignments were done with the programs PILEUP and PRETTYBOX (25, 50). The default threshold parameters for PRETTYBOX were used: 1.5, identical (black boxes); 1.0, similar (dark grey boxes); 0.5, somewhat similar (light grey); 0.0, dissimilar (white boxes) (see Fig. 2). Table 2 was constructed by using data generated from the program OLD_DISTANCES (25, 50).

#### Subcloning and construction of an *H. ducreyi* mutant

The plasmid pool was subcloned by removal of two small HindIII fragments by restriction and religation of the gel-purified 6.5-kb fragment to form pUNCH 563 (Fig. 1). pUNCH 563 was mutagenized by restriction with Bsm1I and MlAI followed by Klenow enzyme treatment to create blunt ends (Fig. 1). A chloramphenicol-resistant clone isolated from plasmid pNC40 (61) by digestion with BgII and end repair using Klenow enzyme. This cassette was ligated to digested pUNCH 563 to form pUNCH 568 (Fig. 1). The deleted DNA between the Bsm1I and MlAI sites of pUNCH 563 contains sequences coding for the C terminus of ExbD, all of ExbD, and the N terminus of TonB. *E. coli* IR754(pUNCH 556) was electroporated into the gel-purified 6.5-kb fragment to form pUNCH 563 (Fig. 1). The inclusion of hgbA mutants EB53 and IR754 were maintained on LB agar plates containing 25 μg/ml 8-aminolevuloinic (8ALA) acid with antibiotic selection where appropriate. Antibiotics were used at the following concentrations for *E. coli*: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 30 μg/ml; and tetracycline, 12 μg/ml. Antibiotic concentrations for *H. ducreyi* were 1 μg/ml (chloramphenicol) and 20 μg/ml (kanamycin).

#### Growth conditions for testing phenotypes

For inoculation of plates for testing phenotypes, *H. ducreyi* strains were starved by growth anaerobically on GCB-I plates without a heme source (56). This method of inoculation reduced the possibility of heme carryover or differences in intracellular heme stores among the various strains. After *H. ducreyi* was grown anaerobically on GCB-I plates, inocula were standardized spectrophotometrically and 10-fold dilutions were made for each strain in a microtiter tray. A Steers replicator was used to inoculate agar medium containing dilutions of each heme source. Thus the inoculum and heme sources were present in a checkerboard titration. Plates were incubated at 35°C in 5% CO2.

#### Outer membrane isolation and analysis

Large-scale cultures of *H. ducreyi* were performed in Fernbach flasks with 1 liter of GCB-I broth containing 5% fetal calf serum (FCS) and heme at 2 μg/ml (heme limiting) or 50 μg/ml (heme replete) (22). It has been shown by several investigators that *H. ducreyi* and *H. influenzae* strains require less heme when grown in liquid medium than when grown in solid medium (3, 22, 60, 64). These strains should not be compared with those used in solid medium used for testing phenotypes. Flasks were inoculated to a starting density of 2.4 × 10⁶ CFU/ml with growth harvested from chloroform agar plates and were incubated 24 h with shaking in a 5% CO2 atmosphere at 35°C. The purity of each culture was routinely verified by streaking onto agar plates which did (chocolate agar) and did not support the growth of *H. ducreyi* but which supported the growth of most other bacteria (GCB-I, without heme). This ensured that minor contamination could be detected and that the novel (previously undescribed) proteins detected (see Fig. 5A) were not the result of contamination. Outer membranes were harvested as previously described (22) except that lysosome was added to the harvested cells prior to the French press step (200 μm, 10 min, 4°C) and two solubilizations were performed with Sarkosyl rather than one. Protein concentrations were determined by using a bicinchoninic acid kit from Pierce (Rockford, Ill.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed as previously described (22) except for the antisera to HgbA. Anti-HgbA was produced by immunizing mice with 25 μg of purified HgbA (22) three times at 2-week intervals. Complete Freund’s adjuvant was used for the first immunization, and incomplete Freund’s adjuvant was used for the last two immunizations.

#### Chemicals and reagents

All chemicals and reagents, unless otherwise noted, were from Sigma.

#### Strains and media

Bacterial strains used in this study are described in Table 1. For routine growth, *H. ducreyi* was maintained on chocolate agar plates prepared by following gonococcal medium base (GCB) instructions (Difco). No fetal bovine serum was utilized in chocolate agar plates used for routine passage. Plates containing the various heme sources were prepared by using GCB and IsoVitalex X supplements (Difco) (termed GCB-I). Human hemoglobin (H7379; Sigma Chemical, St. Louis, Mo.) was dissolved in phosphate-buffered saline at a concentration of 10 mg/ml and rocked at room temperature for 2 h or overnight at 4°C prior to filter sterilization. Hemoglobin (Sigma) was made 10 mg/ml in 0.1 N NaOH and was used without sterilization. To bind hemin to either serum or serum proteins, various amounts of heme were added to the protein source and allowed to bind for at least 10 min at room temperature prior to incorporation into GCB-I plates. Human serum albumin (HSA; Sigma) was saturated to 50% by the addition of 1/2 mol of heme per mol of albumin. We assumed that the albumin contained no heme to start with and that it contained a single heme binding site. Catalase was obtained from Sigma (C-100, lot no. 106H7055; C-6655, lot no. 23H70355). Five micrograms of hemin per milliliter corresponds to 7.6 μM heme.

*E. coli* hema mutants EB53 and IR754 were maintained on LB agar plates containing 25 μg/ml 8-aminolevuloinic (8ALA) acid with antibiotic selection where appropriate. Antibiotics were used at the following concentrations for *E. coli*: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 30 μg/ml; and tetracycline, 12 μg/ml.
FIG. 1. Restriction maps and phenotypes of relevant *H. ducreyi* *exbB*, *exbD*, and *tonB* clones. Not shown in pUNCH 568 are unique *Xba*I and *Xho*I vector sites used to release the insert prior to allelic exchange. *E. coli* IR754 (*hemA* *tonB* *aroB*) containing pUNCH 556 and the indicated plasmid was inoculated onto LB medium containing various sources of heme or δALA: hemoglobin (Hg), 100 μg/ml (6 μM hemin); heme, 50 μg/ml (77 μM) or 10 μg/ml (15 μM); heme-HSA (H-Alb), 3,300 μg/ml (50 μM), saturated at a 50% molar concentration with hrem, 16 μg/ml (25 μM); δALA, 25 μg/ml (4 μM). +, macroscopic growth at 48 h; −, no macroscopic growth at 48 h.

Plasmids

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<th>Heme</th>
<th>H-Alb</th>
<th>δALA</th>
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<td>−</td>
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</table>

*pUNCH 562* 1.1 kb

*pUNCH 563* 1.5 kb

*pUNCH 568* 3.5 kb

*pUNCH 565* (hgbA)

*pUNCH 556* (hgbA)

*pUNCH 555* 1.5 kb

*pUNCH 556* (hgbA) without

RESULTS

Cloning of the *H. ducreyi* *exbB*, *exbD*, and *tonB* loci. We found that cloned *hgbA* was able to confer upon *E. coli* EB53 (*hemA* *aroB*) (Table 1) the ability to bind human hemoglobin (23), but this strain was unable to utilize hemoglobin as a source of heme (data not shown). We reasoned that HgbA expressed in *E. coli* EB53 might not be energized by the *E. coli* Ton system, since *E. coli* and *H. ducreyi* are only distantly related (2). An experiment was designed to clone the Ton system of *H. ducreyi* which was similar to that used to clone the Ton system of meningococci (57). A partial *Sau*IIIAl library was transformed into *E. coli* IR754 (*tonB* *hemA* *aroB*) expressing *hgbA* from the compatible plasmid pUNCH 556 (Table 1) and plated on hemoglobin as the sole source of heme. If the hemoglobin receptor plus gene products from certain plasmids allowed the uptake of heme from hemoglobin, then the *E. coli* heme synthesis mutation would be bypassed and growth on hemoglobin plates would occur. Of approximately 5 × 10⁶ total transformants, 30 complementing clones were obtained on LB hemoglobin plates. To confirm that these clones required HgbA for growth on hemoglobin, plasmid DNA isolated from each was transformed into *E. coli* IR754 lacking pUNCH 556 (hgbA) and again plated onto hemoglobin agar plates. Thirteen of the 30 clones failed to grow without pUNCH 556, suggesting that the gene products expressed from those clones were insufficient to allow IR754 to utilize the heme from hemoglobin. This screen eliminated clones which allowed the heme from hemoglobin to leak in nonspecifically or directly complemented the *hema* defect in IR754. Eleven of the 13 plasmids hybridized to the insert from pGJ300 containing the *H. influenzae* *tonB* operon (33, 34) (data not shown). The two plasmids which did not hybridize apparently suffered deletions during propagation since they were reduced to the size of the vector. Nine of 11 hybridizing plasmids contained a 6.5-kb hybridizing

Nucleotide sequence number. Relevant DNA sequence data of the Ton system gene locus has been submitted to GenBank (accession no. AF001034).
band; each conferred upon IR754 carrying pUNCH 556 (hgbA) the ability to grow on LB hemoglobin agar (data not shown).

One plasmid, pUNCH 562, was chosen for further study (Fig. 1).

DNA and deduced amino acid sequence of the H. ducreyi Ton system gene cluster. The location of the TonB gene cluster was revealed by subcloning and deletion analysis as shown in Fig. 1. The relevant regions were sequenced, and the order of the genes was exbB exbD tonB. Sequences similar to -35 and -10 consensus sequences were found 114 and 82 nucleotides (nt) upstream of the exbB start codon and were separated by 16 nt. Putative ribosome-binding sites were found at 9, 11, and 11 nt upstream of the respective exbB, exbD, and tonB ATG start codons. Just downstream of the tonB open reading frame there was an AT-rich region with dyad symmetry consistent with a transcription terminator. Notably absent were sequences corresponding to promoters upstream of the exbD and tonB structural genes.

Comparisons of the H. ducreyi deduced amino acid sequences with the ExbB, ExbD, and TonB protein sequences are shown in Table 2. The amino acid sequences for ExbB and ExbD were most similar to the proteins from other members of the pasteurellae, and this family comprised a group separate from the others. The ExbB and ExbD proteins were the most similar...
among all the genera, whereas the sequences of the TonB proteins were more divergent. The TonB protein of *H. ducreyi* was most closely related to the TonB protein from *Pasteurella haemolytica* (26).

**Phenotypic characterization of the *H. ducreyi* Ton system.**

Subcloning and deletion analyses were used to define regions of the original plasmid pUNCH 562 required to express a hemoglobin-utilizing phenotype. *E. coli* IR754(pUNCH 556) containing subclone pUNCH 563 also demonstrated a hemoglobin-utilizing phenotype (Fig. 1 and 3A), but not in the absence of pUNCH 556 (*hgbA*) (Fig. 1). pUNCH 563 was mutagenized by deleting the DNA between the unique BbsI and MluI sites and replacing it with a Cmr (23) cassette to form pUNCH 568 (Fig. 1). This deletion included all of *exbD* and portions of *exbB* and *tonB*. IR754(pUNCH 556) containing the mutagenized Ton system plasmid pUNCH 568 was unable to grow on hemoglobin (Fig. 1). Previously, we reported that *E. coli* clones expressing *hgbA* (pUNCH 556) are somewhat leaky in that the outer membrane is perturbed (the periplasmic enzyme RNase leaks to the extracellular environment) (23, 69). We assume that this leakiness accounts for the strain’s ability to grow on high levels of hemin. Neither IR754(pUNCH 556) containing pUNCH 563 nor IR754(pUNCH 556) containing pUNCH 568 grew on 50 μM HSA (3.3 mg/ml) saturated at 50% hemin (Fig. 1). Fifty micromolar HSA is about 10% of the normal serum concentration. All strains grew on medium containing 6ALA, confirming viability. Thus, these data indicated that under these conditions, energized HgbA functioned as a.
chloramphenicol-containing chocolate agar. To screen for pu-

with deletion/mutant plasmid pUNCH 568 and plated on

moglobin (200 μg/ml) [12 μM hemin] or chocolate agar and incubated at 35°C

for 48 h with 5% CO₂.

An H. ducreyi Ton system mutant was constructed to examine

which sources of heme are utilized by TonB-dependent

receptors. Parent H. ducreyi strain 35000 was electroporated

with deletion/mutant plasmid pUNCH 568 and plated on

chloramphenicol-containing chocolate agar. To screen for

putative mutants, 10 Cm⁺ transformants were tested for the ability
to grow on hemoglobin agar, and none was able to. Shown in

Fig. 4 is the phenotype of one transformant, FX514 (ΔexbB exbD tonB); it was used for further experiments. Positive control

care strain FX504 (hgbA) grew on hemoglobin, and negative

control strain FX504 (hgbA) did not.

Evidence that the inability of FX514 to utilize hemoglobin

was due to a specific mutation in the Ton system was obtained

as follows. The mutation in the FX514 chromosome was re-

paired by electroporating Ton system plasmid pUNCH 563

followed by selection on hemoglobin agar (Tables 1 and 3).

Since plasmid pUNCH 563 cannot replicate in H. ducreyi, growth on hemoglobin selected for repair of the mutant Ton

system chromosomal locus. In addition, the defect in FX514

was complemented in trans by using pUNCH 1210 (Table 3).
pUNCH 1210 contains the entire Ton system insert from

pUNCH 563 in shuttle vector pLS88, which is able to replicate

in H. ducreyi (Table 1). Since both chromosomal repair and

complementation in trans restored the ability of the H. ducreyi

FX514 to grow on hemoglobin, but vector control plasmid

pLS88 did not, we concluded that the inability of FX514 to

grow on hemoglobin was due to a specific mutation in the Ton

locus (data not shown). Furthermore, the phenotype of FX514

was not due to a polar effect on downstream genes since plas-

mid pUNCH 1210 fully restored hemoglobin utilization.

Southern blotting of chromosomal DNA from FX514 con-
firmed that FX514 contained the appropriately larger HincII-
to-KpnI fragment (3.25 kb) compared to parent 35000 (3.0 kb)

(Fig. 1 and Southern blot data not shown). The HincII-to-KpnI

fragment in FX514, but not 35000, was recognized by the CAT

probe. The appropriately sized HincII-to-KpnI bands were also

TABLE 2. Percent identities among ExbB, ExbD, and

TonB homologs

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<th>Strain</th>
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* Hd, H. ducreyi; Ph, P. haemolytica; Hi, H. influenzae; Nm, Neisseria meningitidis; Pp, Pseudomonas putida; Ec, E. coli.

* a Calculated by pairwise comparison of the sequences aligned in Fig. 2, using OPEEDISTANCES (25, 50). The percentage is the number of identical residues divided by the length of the shorter sequence without gaps.

** TABLE 3. Growth of H. ducreyi on agar plates containing various heme sources **

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hemo-</th>
<th>Hemin</th>
<th>Heme-albumin</th>
<th>Catalase</th>
<th>Chocolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>35000 (wild type)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FX504 (hgbA)</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>FX514 (exbB exbD tonB)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>FX514(pUNCH 1210)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>FX514 repair</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* a, +, macroscopic growth present by 48 h; --, no macroscopic growth at 48 h.

b Concentration (micrograms per milliliter).

c The Ton system mutation in FX514 was repaired to wild type as described in text.

FIG. 4. Growth of H. ducreyi Ton system isogenic mutant on hemoglobin agar. H. ducreyi parent strain 35000 (wild type [wt]), FX504 (hgbA), and FX514 (ΔexbB exbD tonB) were streaked onto GCB-I medium containing human hemoglobin (200 μg/ml [12 μM hemin]) or chocolate agar and incubated at 35°C for 48 h with 5% CO₂.

FIG. 5. SDS-PAGE and Western blotting of outer membranes from H. ducreyi. The indicated strains were grown under heme-replete (+) or heme-limiting (−) conditions, and their outer membranes were isolated and subjected to SDS-PAGE (7.5% gel) and Coomassie staining (A) or Western blotting using anti-HgbA sera (B). Strains were H. ducreyi parent strain 35000 (wild type), FX504 (hgbA), and FX514 (ΔexbB exbD tonB). Positions of molecular mass markers are shown at the left. HgbA, hemoglobin receptor protein; protein bands 1, 2, and 3, previously undescribed heme-regulated proteins expressed by Ton mutant FX514 (protein 2 refers to the lower band of the doublet). The Western blot (B) was purposely overloaded in order to detect potential proteolytic breakdown products of HgbA as a source of proteins 2 and 3 and other, smaller proteins.
observed in pUNCH 563 and pUNCH 568, respectively, using these probes (data not shown). These results indicated FX514 contained an allelic replacement of the Ton system. Chromosomal repair of FX514 resulted in a Southern blot pattern identical to that of 35000.

**Phenotype of the H. ducreyi Ton mutant on sources of heme other than hemoglobin.** In other bacteria, Ton system mutations are impaired in the ability to use a variety of heme/iron compounds transported by TonB-dependent receptors; therefore, the growth phenotype of FX514 was examined on other heme-containing media that support the growth of H. ducreyi. H. ducreyi was first heme starved (56) and then serially diluted for use as the inoculum. There was no difference for either mutant on hemin or catalase relative to the parent (Table 3).

All three strains grew with hemin at 50 \( \mu \text{g/ml} \) but not lower amounts. None of the three strains grew on GCB-I agar containing 33 \( \mu \text{M} \) HSA (2 mg/ml) saturated with 50% hemin. Taken together, these data indicated that utilization of hemoglobin was TonB dependent whereas utilization of free hemin and catalase appeared to be TonB independent.

**Novel proteins expressed by H. ducreyi Ton system mutant FX514.** Previous work in *E. coli* demonstrated that Ton system mutants are relatively iron starved based on the observation that genes normally regulated by Fur are derepressed (48). SDS-PAGE analysis of outer membrane proteins from *H. ducreyi* Ton mutant FX514 demonstrated several heme-regulated gene products present in increased amounts (Fig. 5A). The major protein present in increased amounts in FX514 was HgbA, confirming that the inability to utilize hemoglobin in this mutant was not due to the lack of synthesis of HgbA. Three other outer membrane proteins, designated 1, 2 (protein 2 refers to the lower band of the doublet), and 3 in Fig. 5A, were also present in increased amounts in certain of the mutants under heme-regulated conditions. Protein 1 was present only in FX514 grown under heme-limiting conditions. Proteins 2 and 3, although present in strain 35000, were more highly expressed in the two mutants than in strain 35000 under heme limitation. In experiments not presented here, Western blotting of *H. ducreyi* using specific antibodies to proteins 2 and 3 demonstrated they were clearly heme regulated, albeit at lower amounts than HgbA (data not shown). Outer membranes prepared from FX514 which had been repaired by chromosomal transformation regained the protein expression pattern of parent strain 35000 (data not shown).

In some gels, including Fig. 5A, two minor proteins of approximately 60 and 65 kDa were present in slightly increased amounts in 35000 and FX514 grown under heme limitation. It was possible that these lower-molecular-weight protein bands present only in 35000 and FX514 were breakdown products of HgbA, since they were absent in FX514. Immunologically reactive material in Western blots of *H. ducreyi* in this size range have been occasionally observed in assays using antiserum to HgbA (data not shown). To address this issue, a Western blot of these preparations was probed with polyclonal antiserum to purified HgbA (Fig. 5B). The gel used for this blot was intentionally overloaded to visualize minor breakdown products of HgbA. Besides the expected reactivity to HgbA, reactivity to protein 1, but not protein 2, protein 3, or smaller bands present in strain 35000 or FX514, by this antiserum was observed. These data are consistent with derepression of the several genes encoding heme/iron-regulated outer membrane proteins.

**DISCUSSION**

**Utilization of hemoglobin is TonB dependent.** Several TonB-dependent receptors for heme or hemoglobin have been cloned from gram-negative pathogens, utilizing a strategy based on the complementation of a heme synthesis defect (*hemA*) or iron acquisition (*aroB*) defect in *E. coli* (20, 29–31, 42, 57–59, 62). This method relies on the principle that *E. coli* K-12 strains are unable to transport hemin or hemoglobin across the outer membrane. However, once heme traverses the outer membrane, *E. coli* K-12 strains are able to transport it across the cytoplasmic membrane, where it can be used to fulfill its heme or iron requirements. Using this method, we have also attempted to clone a heme receptor from *H. ducreyi* in *E. coli* EBS53 (*hemA* aroB), but initial experiments have been unsuccessful. This failure, coupled with our inability to demonstrate a TonB-dependent hemoglobin utilizing phenotype in *E. coli* EBS53 with cloned *hgbA*, prompted us to search for an explanation. It was found that the cloned *H. ducreyi hgbA* receptor required its homologous Ton system in *E. coli* for growth on hemoglobin. Furthermore, the Ton system mutant FX514 was unable to utilize hemoglobin at wild-type levels, unambiguously demonstrating that HgbA was TonB dependent.

Since the deletion plasmid used to construct the *H. ducreyi* Ton system mutant contained deletions in *exbB, exbD*, and *tonB* genes, it is not possible to infer if the Ton system could function in the absence of only a single protein. However, it should be noted that in *E. coli*, most null mutations in any three of these genes result in the total or partial loss of function for the Ton system.

**Role of HgbA in heme utilization.** In an experiment to examine the role of HgbA in heme acquisition, we tested whether cloned HgbA with its homologous Ton system could confer upon *E. coli* hemA tonB aroB strains the ability to grow on free hemin or hemin complexed to HSA. No growth was observed on HSA-heme agar. Growth on hemin agar was observed only at high concentrations (50 \( \mu \text{g/ml} \)) and growth was TonB independent, suggesting that HgbA alone does not function as a typical TonB-dependent receptor for free hemin or hemin complexed to HSA. This finding does not rule out the possibility that HgbA is involved in internalization of free hemin in *H. ducreyi*, but may indicate that additional components are required. Alternatively, HgbA and other (heme/iron) receptors may form a complex, and the absence of HgbA may disturb the function of this complex. Similar nonspecific defects in the uptake of iron compounds have been found for TonB-dependent receptor mutants in other bacteria (1, 6).

**Utilization of heme or catalase in *H. ducreyi* is TonB independent.** Although neither *H. ducreyi* FX504 nor FX514 grew on hemoglobin as a sole source of heme, both mutants exhibited no such growth defect on hemin relative to parent 35000 when prestarved for heme by growth anaerobically in the absence of heme. Further, all three strains required relatively high concentrations of free hemin (50 \( \mu \text{g/ml} \)) for growth on plates. To date, bacteria containing documented heme receptors require much less heme (less than 10 \( \mu \text{g/ml} \)) than *H. ducreyi* to fulfill their iron requirement. The requirement of the wild-type *H. ducreyi* strain for high concentrations of heme is reminiscent of certain mutants of *H. influenzae*. *H. influenzae* tonB (34) or hxuC (17) mutants require high levels of heme (50 \( \mu \text{g/ml} \)), whereas wild-type strains require only 10 \( \mu \text{g/ml} \) or less (0.1 \( \mu \text{g/ml} \)) (60). Most previous studies have either shown the TonB dependence or implied the TonB dependence of heme uptake (20, 29–31, 42, 57–59, 62). However, in contrast to these previous studies, in *Neisseria* spp., heme utilization has very
recently been shown to be TonB independent (7, 57). No heme receptor has been found in Neisseria spp.

The growth of H. ducreyi on catalase was also TonB independent. We confirmed the ability of H. ducreyi to grow on catalase by using highly purified preparations (Sigma C-100), which demonstrated a single band by SDS-PAGE. It remains unclear whether a specific receptor for catalase exists or whether catalase releases hemin upon prolonged incubation; however, a comparison of the appearance (color) of catalase plates with that of hemin plates indicates that there is insufficient free heme present in the former to support the growth of H. ducreyi.

Previous studies have reported that the addition of FCS improves the growth of H. ducreyi (3). The addition of FCS to plates containing dilutions of heme or catalase reduced the requirements of strain 35000 to 1 µg/ml for both heme and catalase (data not shown). However, FCS did not lower the requirements for heme or catalase for mutants FX504 and FX514. These results are difficult to interpret and suggest several possibilities. The simplest explanation is that FCS contains contaminating hemoglobin. Another possibility is that FCS contains a heme/iron source whose utilization requires the expression of both HgbA and the Ton system. It is also possible that FCS enhances the uptake of heme or catalase or expression of heme or catalase receptors. Experiments are currently under way to address these issues.

Our inability to obtain growth of H. ducreyi on heme-albumin is in contrast to previous studies (3, 39). These differences could at least be partially explained by differences in materials and methods (percent heme saturation and inoculation differences) used in the various studies.

The H. ducreyi Ton system mutant expresses novel heme-regulated outer membrane proteins. Regulation of most iron-repressed genes in E. coli is under the control of the global negative repressor Fur (4). In E. coli, derepression of the Fur regulon in tonB mutants is believed to be due the inability to transport iron by means of the various TonB-dependent receptors, resulting in low intracellular iron levels (47). H. ducreyi contains a functional Fur protein (14); however, the H. ducreyi genes regulated by Fur have not yet been identified. In H. ducreyi, expression of hgbA and perhaps hemolytic activity (9, 23) are regulated by the levels of heme in the medium. Since heme and hemoglobin contain iron, it is possible that these heme sources also serve as a source of intracellular iron, thereby indirectly affecting expression of the H. ducreyi Fur regulon. H. ducreyi Ton system mutant FX514 demonstrated increased expression of HgbA as well as several previously undescribed outer membrane proteins, which appeared to be regulated by the levels of heme in the medium. The possibility that these novel proteins are involved in heme/iron acquisition is consistent with observations made in other pathogens. Further work is needed in this area to understand the repertoire of receptors for heme/iron compounds in H. ducreyi and their regulation.

Structure and arrangement of the H. ducreyi Ton system. The arrangement of genes in the H. ducreyi tonB cluster was similar to that in certain other gram-negative bacteria described elsewhere (8, 33, 34), with the order exhB exhD tonB. The arrangement, the DNA sequence, and the proximity of the structural genes suggested that only one promoter was responsible for expression of all three genes. We speculated that these genes may be transcribed as a multicistronic message; however, this remains to be proven experimentally.

Significant diversity exists between the H. ducreyi and E. coli TonB proteins. These differences could account for the inability of the H. ducreyi HgbA receptor to function together with the E. coli EEB53 Ton system. However, three of four general domains previously described for E. coli TonB were present in H. ducreyi TonB (38, 65). The sequence of the N-terminal hydrophobic region (amino acids [aa] 12 to 32) (36) spans the cytoplasmic membrane in E. coli and may interact with ExbB. H. ducreyi TonB contains three of four invariant residues in this N-terminal region compared to E. coli. The second domain traverses the periplasmic space in E. coli and contains the characteristic X-Pro repeat region (aa 63 to 102) and is present in the H. ducreyi sequence. A third short sequence has been implicated in the interaction of TonB with outer membrane receptors and consists of YPARA (aa 160 to 167) in E. coli and YPARE in H. ducreyi. The fourth domain of TonB (aa 199 to 216) in E. coli, predicted to form an alpha helix, is present in all enteric species yet is so diverged in both Haemophilus species that no primary sequence homology exists. Four monoclonal antibodies against E. coli TonB (38) failed to react with H. ducreyi TonB (data not shown), providing further evidence of the dissimilarity between the TonB proteins of E. coli and H. ducreyi. Despite these dissimilarities, the E. coli proton motive force can be transduced through the H. ducreyi TonB to HgbA, suggesting that certain critical interacting domains are present. The reconstituted system in E. coli containing HgbA and the H. ducreyi Ton system also contains ExbB and ExbD proteins from E. coli. It is not clear whether it is the E. coli or H. ducreyi versions of ExbB and ExbD proteins that interact with H. ducreyi TonB.

It has recently been shown that an hgbA (hupA) mutant of strain 35000 (56) is less virulent in a temperature-dependent rabbit model of infection. In this model, lesion scores were smaller and isolation of the mutant from lesions was unsuccessful. We have confirmed this finding by using FX504 (23a) and have similar data from a swine ear model of infection (23b, 32). These results suggest that the acquisition of hemoglobin is vital for this pathogen to survive and produce disease in vivo. We predict that a Ton mutant would have similar or possibly even more profound virulence defects based on its inability to utilize hemoglobin and possibly other heme compounds transported by TonB-dependent receptors.

Our results implicate several novel outer membrane proteins which were more highly expressed in the Ton system mutant and might be additional TonB-dependent receptors to study. Mutagenesis of the genes encoding these putative novel receptors might give insights into their function for the acquisition of heme- or iron-containing compounds utilized by H. ducreyi. Lastly, assuming that additional H. ducreyi TonB-dependent receptors exist for sources of heme other than hemoglobin, we hope to clone appropriate homologous receptors by functional complementation of E. coli IR754 [PUNCH 563] and selecting on appropriate heme sources.

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