Cloning and Characterization of *tdhA*, a Locus Encoding a TonB-Dependent Heme Receptor from *Haemophilus ducreyi*

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Haemophilus ducreyi **is unable to synthesize heme and must acquire it from its only known host, humans. We cloned and sequenced a gene encoding an outer membrane receptor for heme. It was designated *tdhA* (for TonB-dependent heme receptor A) since it was related by sequence homology to the family of TonB-dependent receptors. TdhA was strikingly similar to open reading frame HI0113 from the genome of *Haemophilus influenzae* Rd and also shared homology with five other heme receptors, including HxuC, HemR, HmuR, ChuA, and ShuA, from gram-negative bacteria. An *Escherichia coli hemA tonB* mutant strongly expressing *H. ducreyi tdhA* grew on low levels of heme as a source of heme only when an intact *H. ducreyi* Ton system plasmid was present, formally demonstrating functional TonB dependence. *tdhA* was expressed poorly in vitro by *H. ducreyi* and only under conditions of heme limitation. A survey of *H. ducreyi* revealed that all tested strains but one synthesized small amounts of TdhA in vitro under heme-limiting conditions. Surprisingly, an isogenic mutant of *tdhA* as well as its parent, 35000, both required the same high levels of heme for growth (50 μg/ml [77 μM] on agar medium). This result, together with previous findings, suggests that in vitro, the uptake of heme by *H. ducreyi* is mediated by a TonB- and TdhA-independent mechanism, possibly diffusion.**

Haemophilus ducreyi is the etiologic agent of chancroid, a genital ulcer disease transmitted by sexual contact (reviewed in references 1 and 49). Chancroid has gained importance recently because it has been implicated as an independent risk factor for the heterosexual transmission of human immunodeficiency virus in Africa (18, 24, 35, 49, 50).

H. ducreyi is characterized by its fastidious and slow-growing nature. *H. ducreyi* is unable to synthesize heme and must obtain heme compounds from its only known host, humans. In vitro, *H. ducreyi* is characterized by an inordinately high heme requirement (50 μg/ml; 77 μM) relative to other bacterial species which express heme receptors.

Host iron is sequestered by several mechanisms, and invading bacteria must gain access to these iron sources to survive and initiate disease. Some bacteria utilize iron-scavenging siderophore systems; others directly bind host iron or heme- or iron-containing compounds such as transferrin, lactoferrin, heme, and hemoglobin (9, 31). *H. ducreyi* does not make siderophores (26). Since *H. ducreyi* requires both heme and iron, differentiation between the requirements for both of these can be problematic.

It is well-established that hemoglobin, hemoglobin complexed to haptoglobin, catalase, and free hemin are able to fulfill the heme requirement of *H. ducreyi* (2, 26, 48). Extracellular heme is complexed by human serum hemopexin or human serum albumin. Whereas it is clear that *H. ducreyi* is unable to utilize heme-hemopexin (11, 20), it is unclear whether it can utilize heme-albumin (14, 26). With the possible exception of the *H. influenzae* system, the form(s) of heme recognized by heme receptors of gram-negative bacteria is not well understood. Heme sources for *H. ducreyi* could be released from host

cells by the action of the *H. ducreyi* hemolysin (33, 34, 47) or cytotoxin (7, 25, 37). In acquiring heme from host heme sources, *H. ducreyi* also fulfills its iron requirement since the heme moiety contains iron.

Most receptors for host iron-binding compounds and siderophore receptors belong to a family of outer membrane receptors designated as TonB dependent because they require the cytoplasmic membrane protein TonB for internalization of bound ligand (3, 5, 43). TonB together with its accessory proteins ExbB and ExbD is believed to comprise a system for the transfer of energy from the cytoplasmic membrane to outer membrane receptors (4).

Initial studies from this laboratory on the heme and iron acquisition systems of *H. ducreyi* identified a 100-kDa hemoglobin binding protein termed HgbA (12). Molecular cloning of the *hgbA* locus revealed a deduced amino acid sequence similar to that of the family of TonB-dependent outer membrane receptors (13, 39). An *hgbA* isogenic mutant of *H. ducreyi* is unable to bind or utilize hemoglobin as a source of heme but can utilize free hemin, implying that HgbA is not required for the utilization of hemin. The hemoglobin acquisition system of *H. ducreyi* was reconstituted in an *Escherichia coli* heme biosynthetic mutant (IR754 *hemA tonB aroB*) by expressing *hgbA* from one plasmid and expressing the *H. ducreyi* Ton system from a second plasmid (14), which implied that the intact heme moiety is internalized. *H. ducreyi* isogenic mutants in either the Ton system or *hgbA* are unable to utilize hemoglobin. In contrast, growth by the *H. ducreyi* Ton system mutant in heme medium is not impaired relative to that of its parent, suggesting a TonB-independent system for heme uptake (14). The requirement for high levels of heme by *H. ducreyi* in vitro is also consistent with a TonB-independent mechanism of heme uptake.

The objective of this study was to determine if *H. ducreyi* contains a heme receptor. A heme receptor of *H. ducreyi* was cloned by transforming a plasmid library into an *E. coli hemA*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
E. coli K-12		
EB53	*hemA aroB rpoB*	40
IR754	EB53 but *tonB*::Kan[r]	42
DH5αMCR	*recA*1 *mcrA mcrBC*	Bethesda Research Labs
H. ducreyi		
35000	Wild type, parent strain; can utilize low levels of hemoglobin and high levels of heme	Stanley Spinola, Indiana University
FX504	35000 *hgbA*::CAT, unable to utilize hemoglobin	13
FX514	35000 Δ(*exbB exbD tonB*)::CAT, unable to utilize hemoglobin	14
FX515	35000 Δ*tdhA*::CAT, can utilize low levels of hemoglobin and high levels of heme	This work
Plasmids		
pBluescript(pBS)	ColE1 replicon, Ap[r], high-copy cloning vector	Stratagene
pNC 40	ColE1 replicon, Ap[r] Cm[r], source of CAT cassette for making insertional mutations	45
pMCL 200	p15A replicon, Cm[r], low-copy cloning vector	30
pHP 45	Omega interposon, source of Str[r]/Spc[r] cassette for making insertional mutations	36
pUNCH 579	*hgbA* in pBS, Ap[r], expresses functional *hgbA* from own promoter	13
pUNCH 563	*exbB exbD tonB* in pBS, Ap[r], expresses functional *H. ducreyi* Ton system from own promoter	14
pUNCH 569	*exbB exbD tonB*, 3.5-kb *Xba*I-*Xho*I of pUNCH 563 in pMCL200	This work
pUNCH 1204	Original *tdhA* clone, 2.9-kb *Sau*3A insert in pBluescript which confers ability of IR754 pUNCH 569 to grow on heme	This work
pUNCH 663	Δ*tdhA*::CAT, CAT cassette of pNC40 (1-kb *Bgl*II, fill-in) between two *Mlu*I sites of pUNCH 1204 (*tdhA*)	This work
pUNCH 665	Δ*tdhA*::Omega, 2-kb *Sma*I from Omega in between two *Mlu*I sites of pUNCH 1204 (*tdhA*)	This work
pUNCH 667	*tdhA*, 2.9-kb *Xba*I-*Xma*I of pUNCH 1204 in pMCL200	This work
pUNCH 676	*tdhA* and *exbB exbD tonB*, 4-kb *Xho*I-*Pvu*I of pUNCH 563 cloned between the *Sal*I and *Pvu*I sites of pUNCH 667	This work

strain expressing the *H. ducreyi* Ton system and plating on heme agar. We have named this *H. ducreyi* heme receptor TdhA for TonB-dependent heme receptor A.

MATERIALS AND METHODS

Strains and media. Bacterial strains used in this study are shown in Table 1. For routine growth, *H. ducreyi* was maintained on chocolate agar plates prepared by following the instructions for gonococcal medium base (GCB) (Difco). Fetal bovine serum (FBS) was not utilized in chocolate agar plates used for routine passage. Plates containing the various heme sources were prepared with GCB and IsoVitaleX (BBL) enrichments (termed GCB-I). Human hemoglobin (catalog no. H7379; Sigma Chemical Co., 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. Heme stock solutions (10 mg/ml) were made by dissolving bovine hemin chloride (Sigma) in 0.1 N NaOH and used without further sterilization.

E. coli hemA mutants EB53 and IR754 were maintained on Luria-Bertani (LB) agar plates containing 25 μM δ-aminolevulonic acid (δALA) with antibiotic selection where appropriate. The following antibiotics were used at the concentrations indicated for *E. coli*: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 30 μg/ml; spectinomycin, 50 μg/ml; and streptomycin, 50 μg/ml.

Growth conditions for testing phenotypes of recombinant *E. coli*. *E. coli* IR754 containing the *H. ducreyi tdhA* and/or Ton system plasmids was used to assess the ability of *E. coli* to utilize heme. After growth overnight at 37°C in LB broth with δALA and antibiotic selection, cultures were centrifuged and resuspended in LB medium with antibiotics but without δALA. Cultures were grown for 1 h to deplete intracellular heme stores and then diluted to an optical density at 600 nm of 0.075 in medium containing indicated amounts of heme or δALA (see Fig. 4).

To determine if internalized hemin could satisfy the iron requirement of IR754 containing the various relevant plasmids (see plasmid constructions below), a disc assay was utilized. IR754 containing either pUNCH 676 (*tdhA* and *exbB exbD tonB*), pUNCH 667 (*tdhA*), or pUNCH 569 (*exbB exbD tonB*) was depleted of heme as described above, 50 μl of this suspension was spread onto agar medium with or without chelators, sterile discs were placed on the agar surface, and heme (10 μl of a 10-mg/ml solution) was pipetted onto the discs. After 24 h at 37°C, growth was recorded. The agar medium used was LB containing 500 μM 2,2-dipyridal. Alternatively, nutrient agar containing either 50 to 200 μM 2,2-dipyridal or 100 μM apoferrichrome A was used.

Growth conditions for testing phenotypes of *H. ducreyi*. *H. ducreyi* strains were heme starved by anaerobic growth on GCB-I plates without a heme source (14, 39). This condition reduced the possibility of heme carryover or differences in

intracellular heme stores among the various strains. Heme-starved *H. ducreyi* 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 inocula and heme sources were present in a checkerboard titration. The heme sources included free hemin, hemoglobin, human serum albumin at 50% heme saturation (assuming a single heme binding site per molecule), pooled normal human serum (pNHS) containing various dilutions of heme, heme containing various dilutions of pNHS, catalase, and FBS containing heme. Plates were incubated at 35°C in 5% CO_2 for 48 h.

Outer membrane isolation, analysis, immunoblotting, and heme-affinity purification. Large-scale cultures of *H. ducreyi* were performed in Fernbach flasks with 1 liter of GCB-I broth containing 5% FBS and 2 μg of heme per ml (heme limiting) or 50 μg of heme per ml (heme replete)(12). Large-scale cultures of *E. coli* were performed in Fernbach flasks containing LB broth and appropriate antibiotics. For induction of iron-stressed conditions in *E. coli*, 2,2-dipyridal (200 μM, final concentration) was added at early log phase, and growth was allowed to continue for 2 h. Outer membranes were harvested as previously described (12). Protein concentrations were determined by using the bicinchoninic acid kit from Pierce (Rockford, Ill.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed as previously described (12).

The antiserum to TdhA was produced as follows. A predicted antigenic peptide was identified from the deduced amino acid sequence of *tdhA* and synthesized by standard fluoroenylmethoxycarbonyl (Fmoc) chemistry by the Microchemical Core Facility, Department of Microbiology and Immunology, University of North Carolina at Chapel Hill. The synthetic peptide sequence was <u>C</u>EFVRRQDRSPTDQD (<u>C</u> functioned for sulfhydral coupling and is not part of the TdhA protein sequence). After purification by reverse-phase high-performance liquid chromatography, the sequence was confirmed by fast atom bombardment-mass spectrometry. The peptide was conjugated to keyhole limpet hemocyanin, and rabbits were immunized a total of five times with 0.5 mg of peptide conjugate per immunization. Freunds complete adjuvant was used for the first immunization and incomplete for the remainder. Immune serum was affinity purified with the TdhA peptide immobilized on a thiopropyl agarose column as previously described (12).

Heme-affinity purification of recombinant TdhA was performed by the procedures of Lee (27, 28) and Yamomoto et al. (51). The difference between these two methods is that in the former method heme agarose from Sigma containing a C_{10} spacer was used and in the latter method heme agarose prepared with a C_2 spacer was used. Outer membranes were used for affinity purification of recombinant TdhA (rTdhA) from recombinant *E. coli* strains (29).
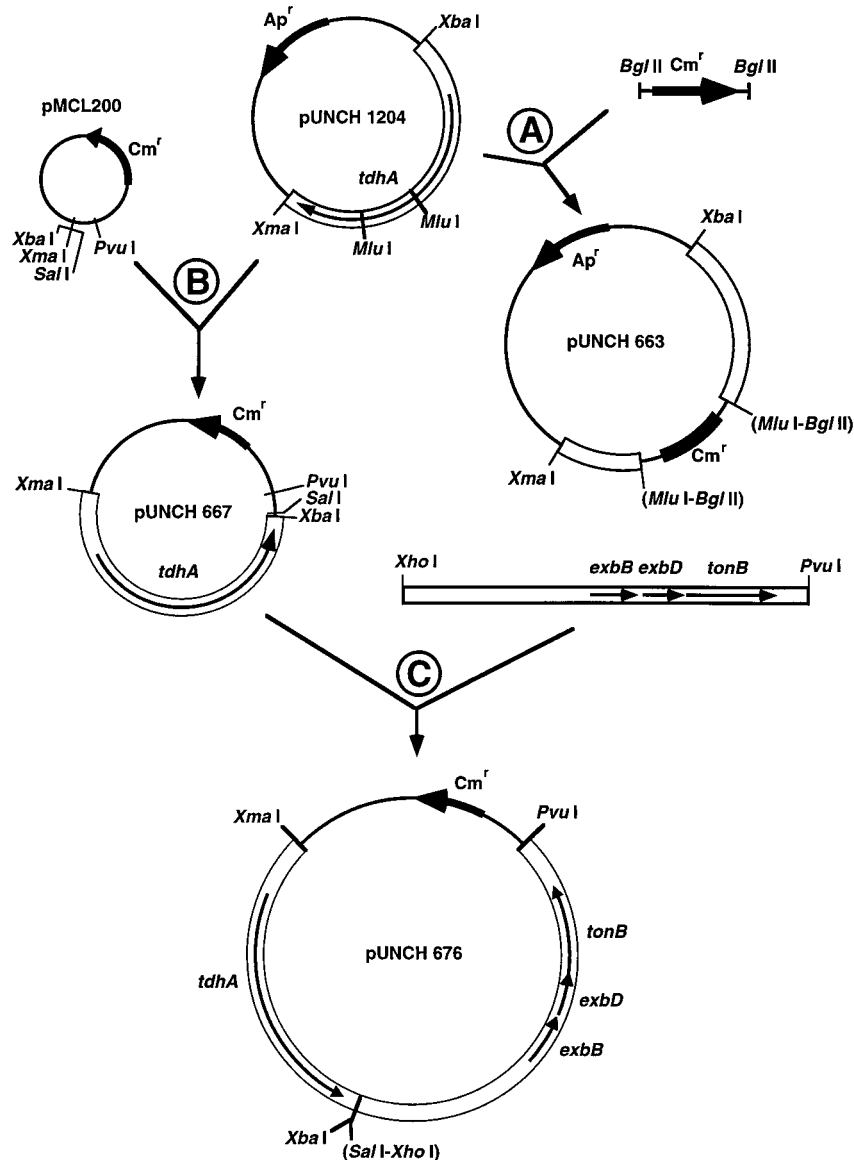
FIG. 1. Construction of relevant *H. ducreyi tdhA* and Ton system (*exbB exbD tonB*) clones. Thick black arrows indicate antibiotic resistance markers; Ap^r and Cm^r indicate the ampicillin resistance and chloramphenicol resistance markers, respectively; thin black arrows indicate relevant genes. Only the restriction sites used are shown; restriction sites in parentheses were predicted to be lost during cloning. The details of cloning steps A, B, and C are described in Materials and Methods.

Chemicals and reagents. All chemicals and reagents, unless otherwise noted, were obtained from Sigma Chemical Co.

DNA manipulations. Standard recombinant DNA methods were used as described in Sambrook et al. (38) or following the manufacturer's instructions. The heme receptor was cloned by using a partially digested *Sau*3AI library from strain 35000 (14). To avoid restriction in subsequent hosts, *E. coli* DH5αMCR was transformed with the ligation mixture, the resulting ampicillin-resistant transformants were pooled, and plasmid DNA was isolated by the Magic Miniprep procedure (Promega). This plasmid pool was electroporated into IR754 (pUNCH 569, *exbB exbD tonB*), and selection was performed on LB agar containing heme (10 µg/ml), ampicillin (100 µg/ml) and chloramphenicol (30 µg/ml). Nine clones were obtained in this fashion, and they were related based on restriction analysis of sites within the inserts (data not shown).

Plasmid constructions. Diagrams of the cloning steps for construction of the new plasmids used in this study are shown in Fig. 1. The *tdhA* gene was disrupted in two separate deletion/insertion clones. The 542-bp *Mlu*I fragment in the coding sequence of *tdhA* was removed and replaced by blunt end ligation with either a chloramphenicol acetyltransferase (CAT) gene from pNC 40 to form pUNCH 663 (Δ*tdhA*::CAT) (step A of Fig. 1) or the omega interposon from pHP 45 to form pUNCH 665 (Δ*tdhA*::Omega). To reduce expression of *tdhA* and potential leakiness caused by overexpression, the *Xma*I-to-*Xba*I insert containing *tdhA* was moved to low-copy vector pMCL 200, resulting in pUNCH 667 (step B

of Fig. 1). Since high-copy expression of the *H. ducreyi* Ton system resulted in reduced growth, the *H. ducreyi* Ton system was subcloned into low-copy plasmid pUNCH 667(*tdhA*) to form pUNCH 676 (*tdhA* and *exbB exbD tonB*) (step C of Fig. 1). This was accomplished by isolating the *Xho*I-*Pvu*I fragment of pUNCH 563 (*exbB exbD tonB*) and ligating it to *Sal*I-*Pvu*I-digested pUNCH 667 (*tdhA*) (*Sal*I and *Xho*I have compatible cohesive ends).

DNA sequencing and analysis. DNA sequence analysis was performed at the University of North Carolina at Chapel Hill Automated Sequencing Facility by utilizing *Taq* terminator chemistry. Both strands were completely sequenced and assembled with AssemblyLIGN software (IBI). Fur boxes were identified by using the Fur consensus oligonucleotide sequence GATAATGATAATCATT ATC and the program DNA Strider. Amino acid alignments were done with the programs PILEUP (17) and SeqVu 1.01 (Garvin Institute, Sydney, Australia). The parameter settings for PILEUP were Blosum, 62; gap weight, 12; and gap length weight, 4. This alignment was then imported into SeqVu.

Construction of an *H. ducreyi tdhA* mutant. An isogenic mutant (FX515 Δ*tdhA*::CAT [Table 1]) was constructed by allelic replacement of the wild-type locus of strain 35000 with the mutation in pUNCH 663 (Δ*tdhA*::CAT). Electroporation of strain 35000 was done with 1 µg of *Xba*I- and *Xho*I-digested pUNCH 663 (Δ*tdhA*::CAT), and selection was performed with 1 µg of chloramphenicol per ml in chocolate agar plates as previously described (13, 19). Chloramphen-
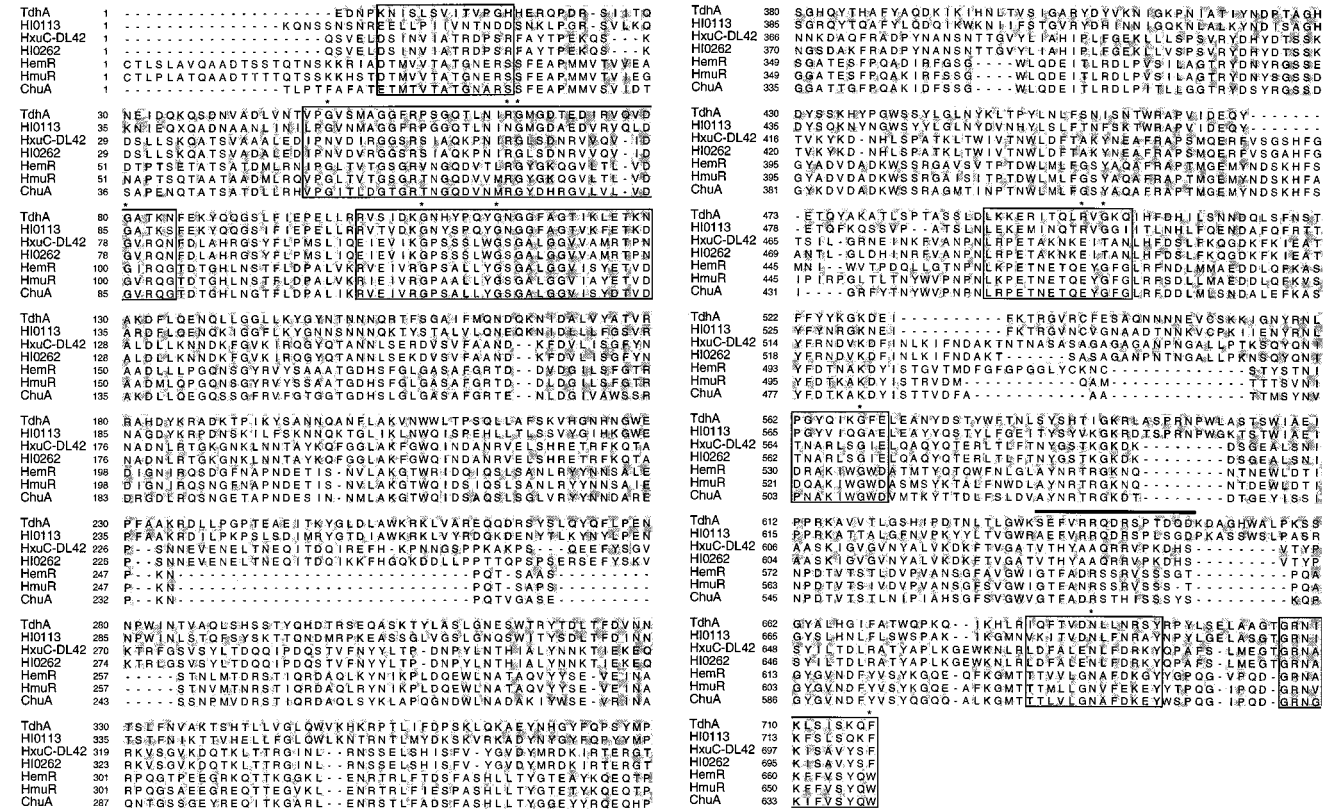
FIG. 2. Comparison of *H. ducreyi* TdhA protein sequence with sequences from heme receptors from other gram-negative bacteria. The shaded boxes indicate residues that scored >75% by a GES matrix with respect to TdhA. The boxed regions indicate areas of conservation found in most TonB-dependent receptors (8). The asterisks indicate highly conserved residues present in TonB-dependent receptors (8). The overlined sequence indicates the residues used to make the peptide antiserum. HI0113, *H. influenzae* Rd ORF 0113 from the genome sequencing project (16); HxuC, heme-hemopexin binding protein from *H. influenzae*, HemR, Heme receptor from *Yersinia enterocolitica*; HmuR, heme receptor from *Y. pestis*; ChuA, heme receptor from enterohemorrhagic *E. coli* EDL933 O:157.

icol-resistant (Cm^r) isolates were tested for the inability to make TdhA by Western blot analysis.

Southern blot analysis was used to confirm that a double crossover occurred in the generation of *H. ducreyi* mutant FX515 ΔtdhA::CAT. Chromosomal DNA was isolated, digested with *Nde*I and *Nru*I, and subjected to electrophoresis and bidirectional transfer. The two blots were probed with either the insert from pUNCH 1204 (*tdhA*) or the *Bgl*II fragment from pNC 40 (CAT cassette). Digoxigenin-labeled, bound probe was detected with alkaline phosphatase-labeled antidigoxigenin antibody (Boehringer Mannheim) followed by detection with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

Nucleotide sequence accession number. The DNA and deduced amino acid sequences of *tdhA* have been submitted to GenBank and assigned accession no. AF052977.

RESULTS

Cloning a TonB-dependent heme receptor. Previously, we reconstructed the hemoglobin acquisition system of *H. ducreyi* in *E. coli* IR754 (*hemA tonB aroB*) (14). This strain of *E. coli* cannot synthesize heme due to the *hemA* mutation and is unable to internalize heme since it lacks hemoglobin and heme receptors. It was observed that the hemoglobin receptor required its homologous Ton system in multicopy in order to remove the heme from hemoglobin and to transport the heme across the outer membrane, demonstrating its TonB dependence. Several TonB-dependent heme receptors from enteric gram-negative bacteria have been cloned in *E. coli* strains containing mutations in heme and/or enterobactin synthesis by plating libraries on hemin agar. In those cases, the heterologous TonB-dependent heme receptor was able to function with the native *E. coli* Ton system. We reasoned that a pu-

tative TonB-dependent heme receptor from *H. ducreyi*, like the *H. ducreyi* hemoglobin receptor, would require its own Ton system. Thus, a partial *Sau*3AI library from *H. ducreyi* 35000 was transformed into *E. coli* IR754 (*hemA tonB aroB*) expressing the *H. ducreyi* Ton system from the compatible plasmid pUNCH 569 (*exbB exbD tonB*) (Table 1) and plated on low levels of heme (10 μg/ml) as the sole source of heme. Nine clones were obtained. To confirm that these clones required the *H. ducreyi* Ton system for growth on heme, plasmid DNA isolated from each clone was separately transformed into *E. coli* IR754 in the presence of pUNCH 569 (*exbB exbD tonB*) or pBluescript (vector control) and again plated onto heme agar plates. All nine grew in the presence of pUNCH 569 (*exbB exbD tonB*) but not pBluescript. This screen was designed to eliminate clones that allowed the heme to leak in nonspecifically or that directly complemented the *hemA* defect in IR754. One plasmid, pUNCH 1204 (*tdhA*), was chosen for further study (Table 1; Fig. 1).

DNA and deduced amino acid sequence of the *H. ducreyi* heme receptor *tdhA*. The relevant region of pUNCH 1204 (*tdhA*) was sequenced and contained a single large open reading frame (ORF) of 2,217 bp which encoded a 739-amino-acid protein. A putative signal peptidase I cleavage site was identified 22 amino acids into TdhA, cleavage of which would result in a mature predicted protein of 81,758 Da. Consistent with the outer membrane localization, TdhA contained a carboxyl-terminal motif ending with a phenylalanine, which is found in the majority of integral outer membrane proteins (44). Beginning
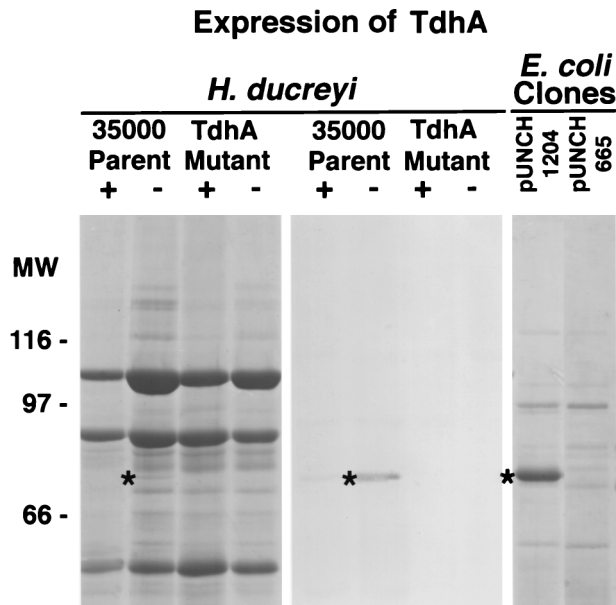
Expression of TdhA

FIG. 3. SDS-PAGE and Western blotting with outer membranes from *H. ducreyi* or *E. coli* expressing recombinant TdhA. *H. ducreyi* strains were grown under heme-replete (+) or heme-limiting (−) conditions. *E. coli* cultures were grown in LB medium in the presence of excess iron. Outer membranes were isolated and subjected to SDS-PAGE (10% total polyacrylamide) and Coomassie staining (outer panels) or Western blotting (middle panel) with anti-TdhA peptide immunoglobulin G. The strains used were *H. ducreyi* parent strain 35000 (wild type), FX515 Δ*tdhA*::CAT, IR754 pUNCH 1204 (*tdhA*), and IR754 pUNCH 665 (Δ*tdhA*::Omega). Molecular weight markers (MW) are indicated in thousands. The asterisks indicate the position of the TdhA protein.

69 and 45 nucleotides (nt) upstream of the *tdhA* start codon, sequences similar to −35 (TGATAA) and −10 (TATATT) consensus sequences were found; these sequences were separated by 18 nt. A putative ribosome-binding site (AAGGAA) beginning 13 nt upstream of the *tdhA* start codon was found. Three putative Fur boxes were found in the putative promoter region beginning 68, 74, and 77 nt upstream of the start codon. Each putative Fur box contained four or five mismatches and no gaps.

A search of the National Center for Biotechnology Information database with the TdhA sequence identified a number of related proteins. The most closely related proteins were a family of TonB-dependent heme receptors from other bacteria (Fig. 2). The amino acid sequence of *H. ducreyi* TdhA was most closely related to an unidentified ORF (HI0113) found in *H. influenzae* Rd. ORFs flanking *tdhA* were similar to HI0174 (5′) and HI0173 (3′) of the *H. influenzae* Rd genome sequence.

TdhA expression in *H. ducreyi* and *E. coli*. To identify the protein encoded by the *tdhA* locus, outer membrane proteins were isolated from *H. ducreyi* 35000, FX515 Δ*tdhA*::CAT, and *E. coli* IR754 containing either pUNCH 1204 (*tdhA*) or pUNCH 665 (Δ*tdhA*::Omega). After SDS-PAGE and Coomassie staining of the gel, parent strain 35000, but not FX515 Δ*tdhA*::CAT, expressed a 75-kDa protein that was barely detectable under low-heme growth conditions (Fig. 3). In contrast, in *E. coli* IR754, a relatively abundant comigrating band was expressed from pUNCH 1204 (*tdhA*) but not pUNCH 665 (Δ*tdhA*::Omega). A corresponding Western blot of the *H. ducreyi* samples probed with anti-TdhA peptide immunoglobulin G (IgG) indicated that these 75-kDa proteins were TdhA (Fig. 3).

TdhA- and TonB-dependent heme utilization. (i) *E. coli*. pUNCH 1204 (*tdhA*) and plasmid pUNCH 665 (Δ*tdhA*::

Omega) were transformed into IR754 pUNCH 569 (*exbB exbD tonB*) and IR754 pMCL200 (vector control) and tested for the ability to grow on LB medium agar containing 10 μg of heme per ml. IR754 pUNCH 569 (*exbB exbD tonB*) was able to grow on heme agar when it contained pUNCH 1204 (*tdhA*) but not pUNCH 665 (Δ*tdhA*) (data not shown). *E. coli* IR754 required both pUNCH 1204 (*tdhA*) and pUNCH 569 (*exbB exbD tonB*) for growth on heme agar. If either plasmid pUNCH 1204 (*tdhA*) or pUNCH 569 (*exbB exbD tonB*) was replaced with a vector control, no growth occurred. *E. coli* EB53 (wild type at the *tonB* locus but *hemA aroB*) containing pUNCH 1204 (*tdhA*) did not grow on heme in the absence of pUNCH 569 (*exbB exbD tonB*) but did in its presence (data not shown). These results demonstrate the requirement for expression of both a functional TdhA heme receptor and a Ton system, both from *H. ducreyi*.

Compared to hemoglobin, heme is a relatively small molecule, which, in theory, could leak across the outer membrane if the outer membrane integrity of IR754 were disrupted due to expression of *tdhA* from pUNCH 1204. To reduce the level of *tdhA* expression and thereby minimize this possibility, low-copy plasmids, each containing *tdhA* and the *H. ducreyi* Ton system singly or in combination, were constructed (Table 1; Fig. 1). Growth curves of IR754 containing *tdhA* and Ton system plasmids singly or together were generated (Fig. 4). *E. coli* IR754 grew in heme broth only in the presence of the plasmid containing both the *tdhA* and Ton system; no growth was observed without heme. Thus, growth on heme was both TdhA and TonB dependent. All strains grew when δALA, which bypasses the *hemA* defect, was supplied, although a decreased rate of growth was observed in the strain expressing both *tdhA* and the Ton system gene products. In order to test for leakiness of the outer membrane to hydrophobic compounds as a potential explanation for growth on heme, discs containing crystal violet, erythromycin, or SDS were placed onto LB δALA agar inoculated with a lawn of IR754 containing *tdhA* and Ton system plasmids. The *tdhA*-expressing plasmids pUNCH 676 (*tdhA* and *exbB exbD tonB*) and pUNCH 667 (*tdhA*) showed zones of inhibition that were slightly larger than the vector control (pMCL200) for crystal violet and erythromycin (4 to 5 mm larger); no differences were observed for SDS. Zone sizes for all three of these compounds were identical for IR754 pUNCH 569 (*exbB exbD tonB*) and IR754 pMCL200 (vector). Thus, both strains which expressed *tdhA* were slightly leaky; however, only the strain which also expressed the *H. ducreyi* Ton system could grow on heme.

We determined whether internalized hemin could be utilized as a source of iron by IR754, pUNCH 667 (*tdhA*), and pUNCH 569 (*exbB exbD tonB*) by incorporating chelators (dipyridal or apoferrichrome A) in the medium and supplying heme on a disc. Growth was observed around the heme disc only in the absence of chelators, demonstrating that additional components may be necessary for efficient removal the iron from heme (data not shown).

(ii) *H. ducreyi*. An *H. ducreyi tdhA* mutant was constructed to examine if heme acquisition was affected by mutagenesis of this locus. The parent strain *H. ducreyi* 35000 was electroporated with deletion mutant plasmid pUNCH 663 (Δ*tdhA*::CAT) (Table 1) and plated on chocolate agar containing chloramphenicol. One *H. ducreyi* transformant, FX515 Δ*tdhA*::CAT, was characterized. Southern blotting of chromosomal DNA confirmed that FX515 Δ*tdhA*::CAT contained the appropriately larger *Nru*I-to-*Nhe*I fragment compared to parent 35000 (Fig. 1) (Southern blotting data not shown). This result indicated that FX515 Δ*tdhA*::CAT contained an allelic replacement of *tdhA*. The growth of *H. ducreyi* mutant FX515 Δ*tdhA*::
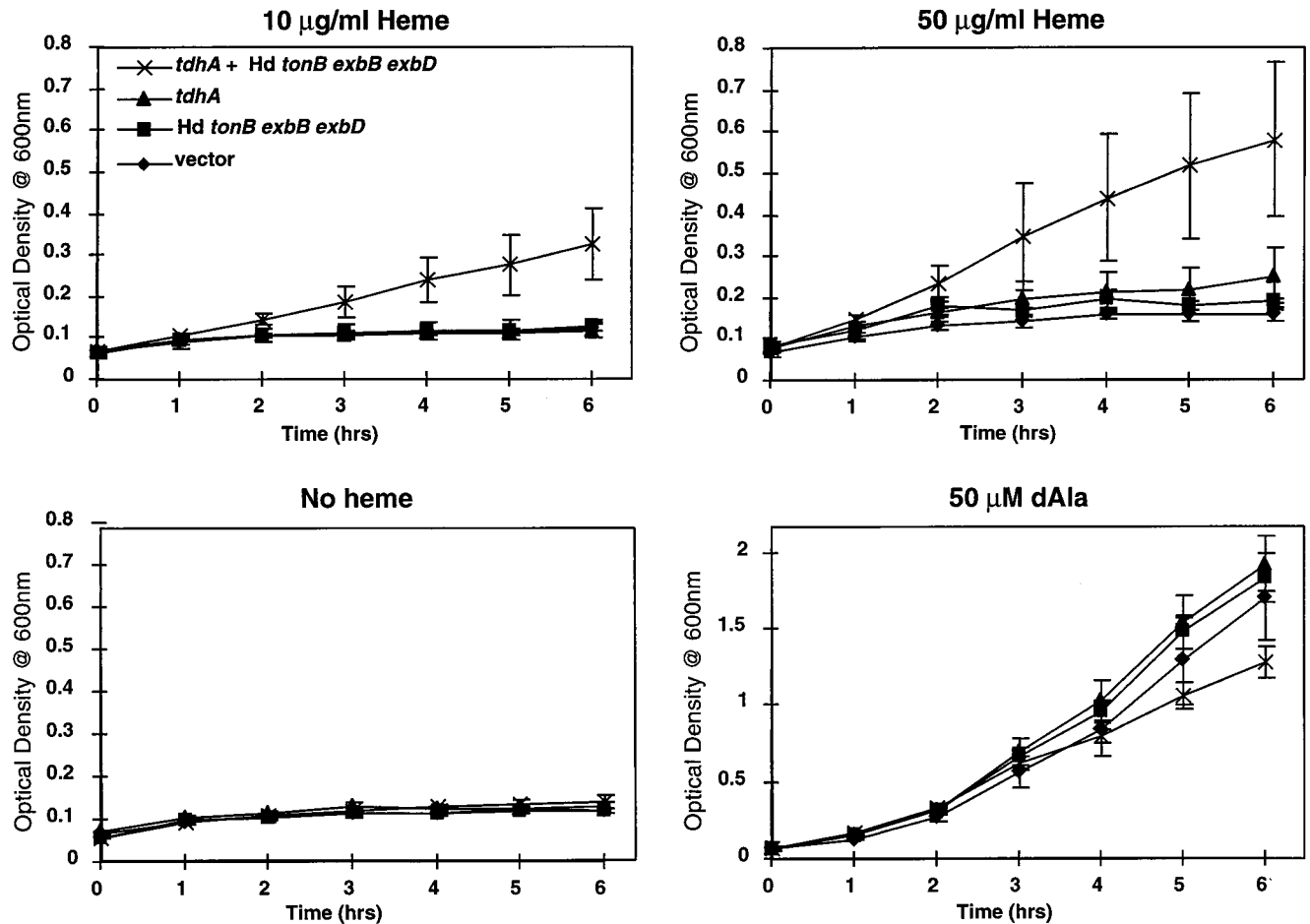
FIG. 4. Growth of recombinant *E. coli* IR754 expressing the *H. ducreyi* heme receptor. *E. coli* IR754 (*hemA tonB aroB*) containing pUNCH 676 (*tdhA* and *exbB exbD tonB*), pUNCH 667 (*tdhA*), pUNCH 569 (*exbB exbD tonB*), or pMCL200 (vector control) was prestarved for heme and inoculated into LB medium containing the indicated heme sources or heme precursors with selection. Note that the scale for the growth curve with the addition of δALA (dAla) differs from that for the other three panels.

CAT was compared to that of the parent strain, 35000, in several heme-containing media. These heme sources included free hemin, 50% heme-saturated heme-albumin, human hemoglobin, pNHS containing variable amounts of heme, catalase, and FBS. No differences in growth were noted (data not shown). Lack of a heme phenotype for mutant FX515 Δ*tdhA*::CAT was similar to that reported for Ton system mutant FX514 Δ(*exbB exbD tonB*)::CAT (14), with the exception that FX514 Δ(*exbB exbD tonB*)::CAT could not grow on hemoglobin.

Affinity purification of TdhA. In several experiments, using a variety of published techniques, attempts to affinity purify a heme receptor from *H. ducreyi* were unsuccessful (data not shown). Since TdhA was expressed at low levels by *H. ducreyi* in vitro, but in relatively large amounts from pUNCH 1204 (*tdhA*) (Fig. 3), we attempted heme purification of rTdhA from *E. coli*. Outer membranes from IR754 containing pUNCH 1204 (*tdhA*) or pUNCH 665 (Δ*tdhA*::Omega) were subjected to heme agarose purification with use of a C_2 spacer as described by Yamamoto et al. (51). Analysis of the heme-binding proteins by SDS-PAGE and Western blotting indicated that rTdhA was the predominant heme binding protein expressed from IR754 pUNCH 1204 (*tdhA*) (Fig. 5). Although other *E. coli* proteins also bound the heme matrix, TdhA was relatively enriched. Since heme is a hydrophobic molecule which
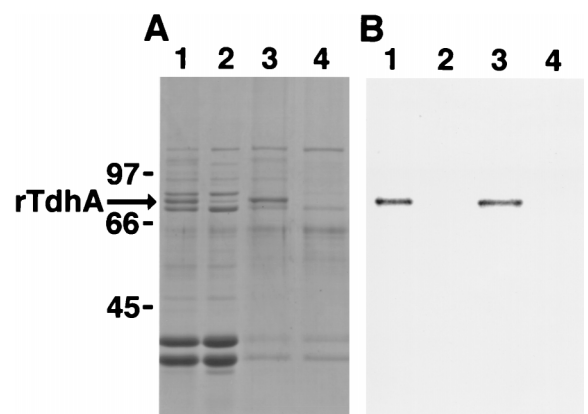

FIG. 5. TdhA binds heme. Outer membranes from *E. coli* IR754 (pUNCH 1204, *tdhA*) and IR754 (pUNCH 665, Δ*tdhA*::Omega) grown under iron limitation were subjected to heme agarose-affinity purification (51) and analyzed by SDS-PAGE followed by Coomassie staining (A) or Western blotting (B) with anti-TdhA peptide immunoglobulin G. Lanes: 1 and 2, 10 µg of outer membrane protein from IR754 pUNCH 1204 (*tdhA*) and IR754 pUNCH 665 (Δ*tdhA*::Omega), respectively; 3 and 4, heme binding proteins from IR754 pUNCH 1204 (*tdhA*) and IR754 pUNCH 665 (Δ*tdhA*::Omega), respectively. The bands immediately above and below TdhA in lane 1 are iron-regulated *E. coli* proteins (compare with Fig. 6, lane OMP). Molecular sizes (in kilodaltons) are indicated on the left.
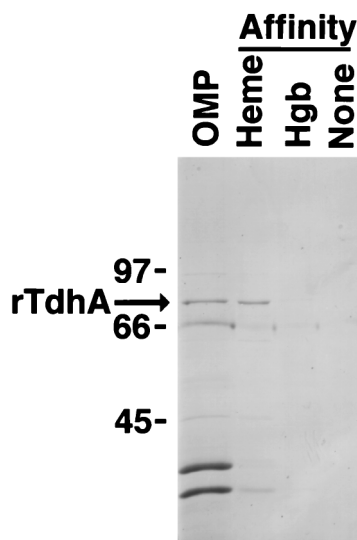
FIG. 6. Binding specificity of rTdhA. Outer membranes from *E. coli* IR754 pUNCH 1204 (*tdhA*) grown under iron excess were subjected to heme agarose-, hemoglobin agarose (Hgb)-, or agarose (no ligand)-affinity purification (51) and analyzed by SDS-PAGE. In the original gel, there was a faint band of TdhA present in the hemoglobin agarose lane, but no band was present in the unsubstituted agarose (no ligand) lane. Molecular sizes (in kilodaltons) are indicated on the left.

could interact nonspecifically with hydrophobic residues found in membrane proteins, we investigated the specificity of TdhA binding to heme. We attempted to use free heme to compete for binding of TdhA to heme agarose. However, the insolubility of hemin at concentrations necessary to achieve heme excess precluded interpretation of these experiments. As an alternative to free heme competition to demonstrate specificity, we used additional alternative solid phases to affinity purify rTdhA (Fig. 6). Outer membranes were mixed with heme agarose, hemoglobin agarose, or agarose that did not contain any ligand and processed as described for heme-affinity purification (51). rTdhA bound well to heme agarose, bound poorly to hemoglobin agarose, and did not bind to agarose lacking a ligand (Fig. 6). Thus, binding of rTdhA to solid phase was specific for heme-containing agarose.

Distribution of TdhA in other *H. ducreyi* strains. In order to determine if other strains of *H. ducreyi* expressed *tdhA*, Western blots of several geographically diverse laboratory and clinical isolates were probed with anti-TdhA peptide immunoglobulin G (Fig. 7). All strains, except the isogenic mutant FX515 Δ*tdhA*::CAT, expressed immunoreactive proteins with similar sizes when the strains were grown under low-heme conditions. In addition to the *H. ducreyi* strains presented in Fig. 7, an additional 26 of 27 strains expressed proteins with similar mobilities which were recognized by this antibody (data not shown).

DISCUSSION

In this paper we have presented three positive lines of evidence that *H. ducreyi* *tdhA* can function as a heme receptor: (i) reconstitution of a functional TonB-dependent heme uptake system in *E. coli*, (ii) sequence similarity of TdhA to five other TonB-dependent heme receptors, and (iii) binding of rTdhA to heme agarose. Additionally, TdhA is synthesized in *H. ducreyi* only under growth conditions of low heme, consistent with, but not proof of, a role in heme or iron uptake. Paradoxically, the *tdhA* mutant lacked a heme phenotype. Possible

explanations for this paradox are that expression of *tdhA* in wild-type *H. ducreyi* in vitro is insufficient, that *H. ducreyi* lacks other components necessary for heme utilization present in *E. coli*, that TdhA functions in *E. coli* differently than in *H. ducreyi*, and that TdhA is not a receptor for heme.

Cloning and TonB dependence of *tdhA*. Several TonB-dependent receptors for heme or hemoglobin have been cloned from gram-negative pathogens by utilizing a strategy based on the complementation of a heme synthesis defect (*hemA*) or iron acquisition (*aroB ent*) defect in *E. coli* (10, 21–23, 29, 40, 42, 43, 46). However, this powerful technique has the important limitation that if the outer membrane of the host is rendered permeable by expression of a heterologous protein, growth on heme may occur. Such leaky growth is characterized by TonB independence and the requirement for relatively high levels of heme. Using similar methods, we have cloned a gene, *tdhA*, encoding a novel heme receptor. Since TdhA required its homologous Ton system to support growth of *E. coli* IR754 on heme, we concluded that it was TonB dependent, at least in *E. coli*. Furthermore, in *E. coli*, TdhA-mediated heme uptake was seen at low (micromolar) heme concentrations, levels consistent with those of other TonB-dependent heme receptors. The leakiness observed for both of the *tdhA*-expressing strains (with and without the *H. ducreyi* Ton system) cannot account for the specific growth on heme by the strain expressing the Ton system, since the additional expression of the Ton system did not make the *E. coli* more leaky.

DNA and deduced amino acid sequence of the *H. ducreyi* heme receptor, *tdhA*. Analysis of the region surrounding *tdhA* revealed that *tdhA* was the only gene implicated in heme transport. In several other systems involved in heme or iron transport, the TonB-dependent outer membrane receptor is flanked by genes encoding other members of the transport system such as a periplasmic binding protein, permease(s), and related ATPases (15, 32, 40, 41). The arrangement of such systems often constitute an operon. The DNA contiguous to *tdhA* did not contain sequences homologous to other genes involved in heme transport. The ORFs surrounding *H. ducreyi* *tdhA* were different from those surrounding the highly related *H. influenzae* Rd ORF HI0113. Heme utilization from heme or hemoglobin would also require transport across the cytoplasmic membrane. *H. ducreyi* and *E. coli* IR754 certainly must contain such a system, but it is yet to be described.

Examination of the deduced amino acid sequence of TdhA revealed homology to those of several TonB-dependent heme receptors from other genera. Homology was found in regions common to TonB-dependent receptors regardless of their ligand specificity, including a putative TonB box near the N terminus of *tdhA* and most of the so-called invariant residues. However, in addition to domains common to TonB-dependent receptors, there were certain residues or very short sequences
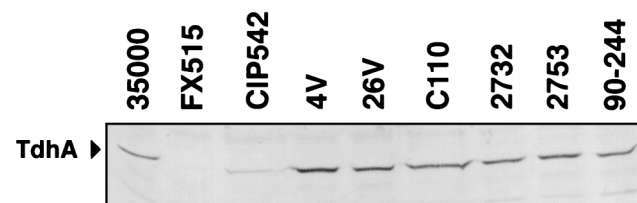

FIG. 7. Distribution of TdhA protein in *H. ducreyi* strains. Geographically diverse *H. ducreyi* strains grown in medium containing low heme were harvested, and total cellular protein (30 μg) was subjected to SDS-PAGE and Western blotting with anti-TdhA peptide immunoglobulin G. The names of strains are indicated above each lane.

scattered throughout the proteins that were common to all the heme receptors (Fig. 2). Longer stretches of sequence similarity were present among the TdhA and HxuC proteins from *H. ducreyi* and *H. influenzae*. We speculate that some of these common residues or sequences could be involved in heme binding or transport.

Expression of the TdhA protein is heme regulated in vitro. In several experiments using *H. ducreyi* 35000, we found *tdhA* to be expressed only under heme-limiting conditions. In contrast to the major heme-regulated protein of *H. ducreyi*, HgbA, TdhA was a very minor protein in Coomassie-stained gels. We estimated that between 50 and 100 times more HgbA than TdhA is made (Fig. 3). Previously, we observed that a Ton system mutant of *H. ducreyi* produced increased amounts of HgbA and TdhA (labeled protein 3 in Fig. 5 of reference 14). These results are consistent with lower intracellular heme or iron levels in the Ton system mutant and subsequent derepression of Fur-regulated genes, as has previously been observed for *E. coli* Ton system mutants.

Three putative Fur boxes were observed in the promoter region of *tdhA*. We hypothesized that the poor expression of *tdhA* in *H. ducreyi* could be due to Fur repression. However, in *E. coli* IR754, *tdhA* expressed from high-copy plasmid pUNCH 1204 resulted in high levels of protein whether or not iron chelators were added to the medium used for growth (data not shown). Furthermore, experiments using *E. coli fur* mutants transformed with high-copy plasmid pUNCH 577 (*H. ducreyi fur* in pBluescript) (6) and low-copy plasmid pUNCH 667 (*tdhA* in pMCL200) failed to provide evidence for Fur regulation of *tdhA* (data not shown). Data suggesting possible *H. ducreyi* Fur regulation include the following: (i) that expression of both *tdhA* and *hgbA* is increased during growth in low levels of heme (iron), (ii) that both *hgbA* and *tdhA* genes contain putative Fur boxes in their promoters, and (iii) that the expression of both genes is increased in Ton system mutants. However, there is currently no direct evidence that Fur regulates these genes. Further studies are required to unravel this interesting regulatory network for *tdhA*, *hgbA*, and other heme regulated genes.

Distribution of TdhA among strains. TdhA was found to be widely distributed in *H. ducreyi* by Western blotting with peptide-specific antisera. The conservation of TdhA among the various strains suggests that it serves an important role, yet its poor expression in vitro suggests that it may be expressed only under certain conditions in vivo. It has been reported that *H. ducreyi* has an extracellular and an intracellular life style and its heme requirement must be met in both environments. Upon lysis of host erythrocytes or nucleated cells by the *H. ducreyi* hemolysin or cytotoxin, it is likely that hemoglobin or heme could be acquired as extracellular sources of heme that could be taken up via their respective receptors. However, in an intracellular environment, it is unlikely that hemoglobin would serve as a source of heme. Thus we speculate that heme synthesis in the cytoplasm of eukaryotic cells could serve to fulfill the heme or iron requirement of intracellular *H. ducreyi*. Within this intracellular environment *tdhA* could be expressed and utilized to exploit this heme source. Studies are underway to address this possibility.

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