INFECTION AND IMMUNITY, June 1995, p. 2194–2200 Vol. 63, No. 6
0019-9567/95/$04.00+0

Characterization of the *hgbA* Locus Encoding a Hemoglobin Receptor from *Haemophilus ducreyi*

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Received 1 December 1994/Returned for modification 8 February 1995/Accepted 22 March 1995

***Haemophilus ducreyi* can bind hemoglobin and use it as a source of heme, for which it has an obligate requirement. We previously identified and purified HgbA, a hemoglobin-binding outer membrane protein from *H. ducreyi*. In this report, we describe the molecular cloning, expression, DNA sequence, and mutagenesis of the structural gene for HgbA, *hgbA*. *H. ducreyi* and recombinant *Escherichia coli* expressing *hgbA* bound [^{125}I]hemoglobin, establishing HgbA as a receptor. Insertions or deletions in the cloned *hgbA* gene abolished expression of HgbA and hemoglobin binding in *E. coli*. Mutagenesis of *H. ducreyi* by allelic exchange of insertions into *hgbA* abolished its ability to bind [^{125}I]hemoglobin or utilize hemoglobin as a source of heme. The deduced protein sequence was similar to those of the TonB-dependent family of outer membrane receptors. The most similar member was HutA (heme receptor) from *Vibrio cholerae*. Tbp1 and Lbp1 (transferrin and lactoferrin receptors, respectively, from pathogenic *Neisseria* spp.) also showed very significant homology. Thus, by characterizing the *hgbA* locus, this work elucidates a potentially important role of HgbA in obtaining heme and/or iron from the host.**

Haemophilus ducreyi is the etiologic agent of the sexually transmitted disease chancroid (1, 17). Chancroid is characterized by ulcerative lesions present on or near the genitals and is sometimes associated with buboes of the inguinal lymph nodes (1, 17). There is an increased incidence of human immunodeficiency virus type 1 (HIV-1) seroconversion for male clients of HIV-1-infected female sex workers who have genital ulcers, suggesting that genital lesions provide a more efficient exit for the HIV-1 virus (23, 31). It has been proposed that the rapid heterosexual spread of HIV-1 in eastern and southern Africa has in large part been due to concomitant genital ulcer disease (24). Thus, eradication of chancroid and other genital ulcer diseases could enhance HIV-1 control programs. At the present time, no vaccine is available and no vaccine candidates are on the horizon.

H. ducreyi is a fastidious, slowly growing, gram-negative rod that is unable to synthesize heme (2). In vitro, *H. ducreyi* must be supplied with heme or heme-containing compounds such as hemoglobin (Hgb) for aerobic growth (2, 13). The in vivo sources of heme are not known; however, *H. ducreyi* is hemolytic (21), which may provide a source of Hgb and therefore a source of heme. An additional uncharacterized step(s) for heme release and internalization remains to be elucidated.

Iron is needed by nearly all bacteria (32). Iron starvation is a major obstacle which infecting bacteria must overcome (19). Some bacteria obtain iron via iron-scavenging siderophore systems (19) or utilize host iron found in transferrin or lactoferrin (4, 20); *H. ducreyi* does neither (13). It is possible that iron contained in heme or Hgb could serve as an iron source for *H. ducreyi* in vivo.

Elkins previously identified and isolated a conserved Hgb-binding outer membrane protein from *H. ducreyi* termed HgbA (6). In this communication, we report the cloning, mutagenesis, expression, DNA sequence, and characterization of the gene encoding HgbA, which we have termed *hgbA*.

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MATERIALS AND METHODS

Bacteria and growth conditions. The *H. ducreyi* type strain, 35000, was obtained from Stanley Spinola, Indiana University, Bloomington, Ind. *H. ducreyi* strains were routinely passaged every 48 h on chocolate agar. For growth of *H. ducreyi* strains under heme-limiting conditions on solid medium (see Fig. 3), bacteria were heavily inoculated onto gonococcal medium base (GC Base; Difco, Detroit, Mich.) supplemented with 1% Isovitelex (Baltimore Biological Laboratory, Cockeysville, Md.) (GCB-I) and either 5 μg of heme per ml (heme deplete) or 50 μg of heme per ml (heme replete) (6).

Cloning of the gene encoding HgbA. Standard methods were followed for molecular biological techniques (16). Oligonucleotides were synthesized at the Lineberger Cancer Center DNA Synthesis Facility at the University of North Carolina at Chapel Hill and were used without further purification. DNA hybridizations were performed with the Genius system according to the directions of the manufacturer (Boehringer Mannheim Corp., Indianapolis, Ind.). Oligonucleotides were labeled with a terminal transferase kit, and plasmid DNA was labeled with a random prime kit (Genius).

The *hgbA* gene was cloned by ligating size-selected *Eco*RI chromosomal DNA fragments from *H. ducreyi* 35000 into *Eco*RI-predigested and prephosphorylated lambda ZAPII arms according to the manufacturer's directions (Stratagene, San Diego, Calif.). After infection and plating of *Escherichia coli* XL1 Blue, the resulting plaques were screened with the degenerate oligonucleotide HgbA1.01 (Fig. 1), which was designed on the basis of the N-terminal amino acid sequence of HgbA (6). Hybridizations and stringency washes with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) were done at 50°C. Positive plaques were subjected to two rounds of purification and excised as Bluescript plasmids according to the directions of the manufacturer (Stratagene). A representative plasmid, pUNCH 579, was chosen for further study. Subclones of pUNCH 579 were made (Fig. 2) and were constructed with the vector Bluescript (Stratagene).

DNA sequencing. Double-stranded sequencing was performed with Sequenase (U.S. Biochemicals) according to the directions of the manufacturer. Both strands of the 5′ region of *hgbA* were sequenced from pUNCH 555 with synthetic oligonucleotides in the authors' laboratory. Subclones pUNCH 553 and pUNCH 557 were subjected to nested deletions with exonuclease III and S1 nuclease, and both strands were sequenced by Lark Sequencing Technologies (Houston, Tex.) (16). To orient subclones, the *Hin*dIII junctions were sequenced (Lark) with pUNCH 579 being used as the template.

Construction of *hgbA* isogenic mutants. The gene encoding chloramphenicol acetyltransferase (CAT cassette) was excised on a 1.6-kb *Hin*dIII fragment from plasmid pNC 40 (28) and used to mutagenize pUNCH 579. This CAT cassette was originally derived from mini-Tn*3* (25). Plasmid pUNCH 579 was partially digested with *Hin*dIII, the linear form was isolated, and it was ligated with the 1.6-kb CAT cassette. Chloramphenicol-resistant transformants were subjected to restriction analysis to identify insertions into each of the three *Hin*dIII sites contained within the insert of pUNCH 579. Insertions were isolated and termed pUNCH 580, 581, and 582 for the A, B, and C *Hin*dIII insertions, respectively, as designated in Fig. 2. *H. ducreyi* was electroporated with pUNCH 580, pUNCH 581, and pUNCH 582, and recombinants were selected on plates containing 1.0

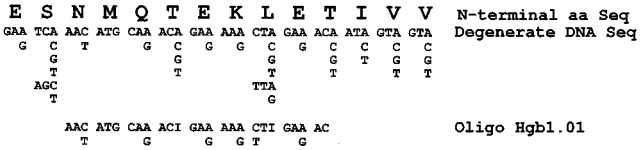

FIG. 1. Synthesis of an oligonucleotide probe. The top single-letter-code sequence (Seq) of amino acids is the N-terminal, 14-amino-acid (aa) sequence from purified HgbA. The middle degenerate sequences are all the possible DNA sequences which could encode the 14 N-terminal amino acids. The lower sequence is the sequence of the degenerate oligonucleotides used in probe Hgb1.01 (64 different oligonucleotides).

μg of chloramphenicol per ml (8). Screening of *H. ducreyi* transformants for expression of HgbA was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting) as described below.

Growth of mutant FX504 on Hgb and human blood agar. In order to test the ability of human Hgb to support the growth of *H. ducreyi* 35000 and the various *H. ducreyi hgbA* mutants, GCB-I agar containing 0, 50, 100, 500, and 1,000 μg of human Hgb (Sigma, St. Louis, Mo.) per ml was prepared as previously described (6). A single colony from a chocolate agar plate was streaked for isolation onto Hgb plates, incubated for 48 to 72 h, and examined for growth. The positive control for growth was a GCB-I plate containing 50 μg of heme per ml, and the negative control was a GCB-I plate lacking heme or Hgb; both were inoculated with wild-type *H. ducreyi* 35000. Determinations of whether human erythrocytes could support extended growth were made by streaking isolated colonies from chocolate agar medium onto GCB-I containing 5% washed human erythrocytes.

Immunoassays and Hgb dot blots. SDS-PAGE (12), Western blots (29), and dot blots (6) were carried out as previously described, with the following exceptions.

For Western blotting of *E. coli* clones, bacteria were grown overnight on Luria-Bertani agar (to avoid problems of instability) containing 100 μg of ampicillin per ml and suspended at an optical density at 600 nm of 0.4. A 1-ml portion of this suspension was pelleted, and the pellet was resuspended in 150 μl of Laemmli sample buffer. This was boiled, and 50 μl of the suspension per lane was loaded on an SDS–7.5% PAGE gel (approximately 25 μg of protein). HgbA antibodies used in this study were those raised against a synthetic peptide based on the N-terminal amino acid sequence of HgbA (6). Since these antibodies recognize the N terminus of HgbA, they would be expected to recognize truncated forms of the protein.

E. coli clones used in the dot blots were grown as described above and suspended at an optical density at 600 nm of 0.4. Portions (100 μl) of the suspension were immobilized onto nitrocellulose. These dot blots were probed with ^{125}I-labeled human Hgb as previously described for *H. ducreyi* (6).

The original screening of *E. coli* clones expressing *hgbA* was done by probing dot blots with biotinylated human Hgb. Human Hgb (Sigma) was biotinylated with a 40:1 molar excess of *N*-hydroxysuccinimidobiotin (Pierce, Rockville, Ill.) to Hgb according to the directions of the manufacturer. Excess biotin was removed by dialysis. The binding of biotin-labeled Hgb was detected in dot blots by probing them with streptavidin conjugated to horseradish peroxidase (Pierce) and developing them with the substrate Enzygraph (Kodak, Rochester, N.Y.). Endogenous peroxidase in *H. ducreyi* prevented the use of peroxidase-containing reagents for *H. ducreyi* dot blots (data not shown).

Nucleotide sequence accession number. The nucleotide sequence presented in this communication has been deposited with GenBank and has been given the accession number U17281.

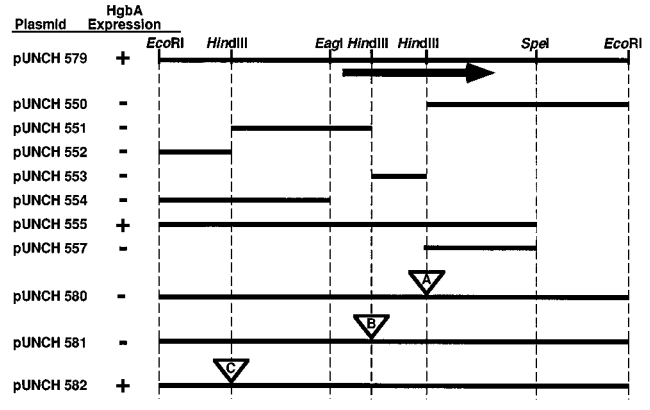

FIG. 2. Restriction map of *hgbA* and flanking DNA. An approximately 8-kb *Eco*RI fragment was cloned into lambda ZAPII and excised as a Bluescript plasmid to form pUNCH 579. Plasmids pUNCH 550 thru 557 are subclones. Plasmids pUNCH 580, 581, and 582 are plasmid pUNCH 579 with a 1.6-kb CAT cartridge inserted into *Hin*dIII sites A, B, and C, respectively. Also shown is a summary of HgbA expression (Fig. 3).

RESULTS

Cloning of the DNA fragment encoding HgbA. On the basis of the N-terminal sequence of HgbA (6), we deduced a degenerate oligonucleotide mixture (termed HgbA1.01) (Fig. 1) and used it to probe separate restriction digests of the chromosome of *H. ducreyi* 35000. In each chromosomal digest, HgbA1.01 hybridized to a single band (data not shown). Digestion of chromosomal DNA from 35000 with *Eco*RI revealed a band of approximately 8.5 kb that hybridized with HgbA1.01. This size is an appropriate size insert for cloning in lambda vectors. The lambda ZAPII system was used to clone the gene encoding HgbA. We probed plaques containing *Eco*RI-digested, size-selected *H. ducreyi* inserts with digoxigenin-labeled HgbA1.01 oligonucleotide and obtained five recombinants which hybridized strongly. These five plaques were excised into Bluescript form. The five recombinants were screened for the binding of biotinylated Hgb to whole cells in a dot blot, and all five bound Hgb; the Bluescript vector control did not bind Hgb (data not shown). Each recombinant contained an 8.5-kb fragment which hybridized in a Southern blot to an identical-sized *Eco*RI fragment in the chromosome of *H. ducreyi* 35000 (data not shown). One clone was designated pUNCH 579 and was used throughout the following experiments. Subclones of pUNCH 579 were made as shown in Fig. 2. Insertions into pUNCH 579 were made by ligation of the antibiotic CAT cassette into each of the three *Hin*dIII sites within the insert of pUNCH 579 in *E. coli* (Fig. 2; also see Materials and Methods).

Expression of HgbA in *E. coli*. Several lines of evidence were sought to determine whether pUNCH 579 contained the structural *hgbA* gene. Western blots were probed with affinity-purified immunoglobulin G antibodies raised against the HgbA N-terminal peptide. The recombinant *E. coli* containing pUNCH 579 expressed a protein which comigrated with native HgbA (Fig. 3A; compare lanes 1 and 3); as expected, the vector control was negative (Fig. 3A, lane 14). Whole-cell dot blots were probed with ^{125}I-labeled human Hgb. Recombinant *E. coli* containing pUNCH 579 expressed an Hgb binding phenotype, but the vector control was negative (Fig. 3B; compare lanes 3 and 14). These data strongly suggested that pUNCH 579 contained the entire *hgbA* gene and that HgbA was a receptor for Hgb. Binding of [^{125}I]Hgb was inhibited by an excess of unlabeled Hgb (Fig. 3C, lane 3), demonstrating that the binding was specific (as discussed below).

Localization of the *hgbA* gene in pUNCH 579. Several strategies were used to localize the *hgbA* structural gene, including hybridization, limited DNA sequencing and Western blotting of subclones, and insertions into pUNCH 579. Hybridization with Hgb1.01 (degenerate oligonucleotide deduced from the N-terminal peptide sequence) localized the N terminus of *hgbA* to a 700-bp *Eag*I-to-*Hin*dIII fragment roughly in the center of the insert contained in pUNCH 579 (Fig. 2). Since just under 3 kb of DNA should be sufficient to encode the 100-kDa HgbA protein, the central location for the N terminus of the gene allowed for either orientation of *hgbA*. Limited sequencing established the orientation of *hgbA* shown in Fig. 2. Western blotting of CAT insertions and subclones was used to confirm the localization and orientation of *hgbA*. Only sub-

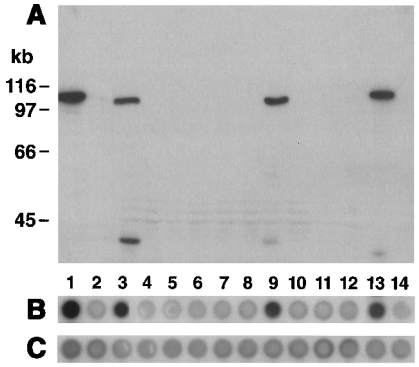

FIG. 3. Western blots and dot blots of *H. ducreyi* and recombinant *E. coli* expressing *hgbA*. *H. ducreyi* and recombinant *E. coli* DH5αMCR containing all of *hgbA*, portions of *hgbA*, or insertions into *hgbA* were subjected to Western blotting (A) or dot blotting (B and C). Western blots in panel A were probed with anti-HgbA. The dot blots in panel B were probed with ^{125}I-labeled human Hgb, and the dot blots in panel C were probed with a mixture of ^{125}I-labeled human Hgb and a 100-fold excess of unlabeled Hgb. Lane 1 is *H. ducreyi* 35000; lane 2 is *H. ducreyi hgbA* mutant FX504. Lanes 3 to 14 are recombinant *E. coli* containing the following plasmids: lane 3, pUNCH 579; lane 4, pUNCH 550; lane 5, pUNCH 551; lane 6, pUNCH 552; lane 7, pUNCH 553; lane 8, pUNCH 554; lane 9, pUNCH 555; lane 10, pUNCH 557; lane 11, pUNCH 580; lane 12, pUNCH 581; lane 13, pUNCH 582; and lane 14, Bluescript vector control. Similar results were seen in at least one other experiment for all strains.

clone pUNCH555 and insertion pUNCH 582 were capable of expressing full-length HgbA (Fig. 2 and 3). On the basis of these data, subclones pUNCH551, pUNCH553, and pUNCH 557 were chosen to complete the sequence of *hgbA*.

DNA sequence analysis and predicted protein. The nucleotide sequence of *hgbA* is presented in Fig. 4. One potential −35 and −10 promoter region is indicated; however, two others exist in this upstream region. A putative Fur box is shown, and it contains within it the −35 sequence. An inverted repeat consistent with a transcription termination signal is just downstream of the open reading frame. A ribosome binding site, AGGAA, is just upstream of the start codon. The open reading frame starts at nucleotide 244 and ends at nucleotide 3159. The open reading frame of 2,919 nucleotides encodes a protein with a calculated molecular weight of 110,936. The predicted protein has phenylalanine at the C terminus, as do most other outer membrane proteins (27). The 14 amino acids obtained by Edman degradation of the purified HgbA protein (6) (Fig. 1) were identical to deduced amino acids 23 to 36, unambiguously confirming that we had cloned the gene encoding HgbA. The first 22 deduced amino acids consisted of a typical signal peptidase I leader peptide and contained the appropriate charged amino acids followed by a hydrophobic region (30). A consensus signal peptidase I cleavage site of AXA was not present

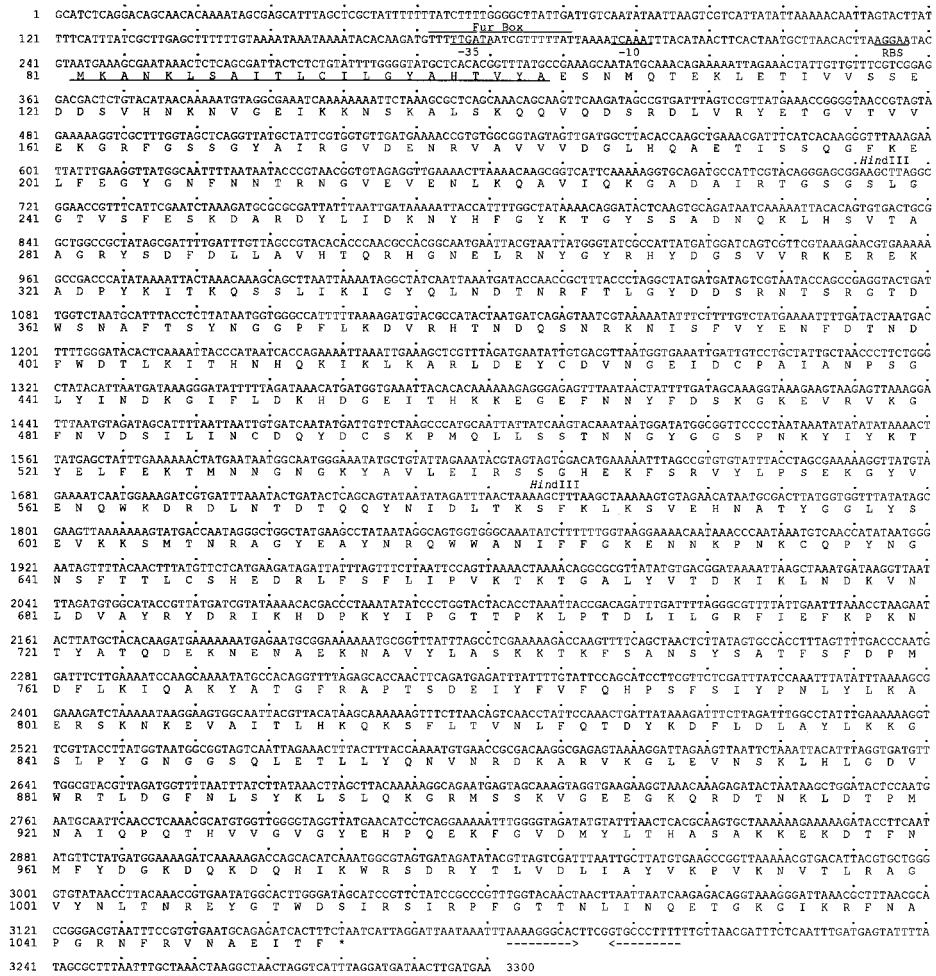

FIG. 4. Nucleotide sequence of *hgbA*. The deduced amino acid sequence is shown in single-letter code below the nucleotide sequence. A putative −10 and −35 promoter sequence, a putative Fur box, and the ribosome binding site (RBS) are indicated and labeled. The signal peptide is indicated by the underlined amino acids. An asterisk indicates the termination codon. Arrows indicate a potential transcriptional terminator stem-loop structure.

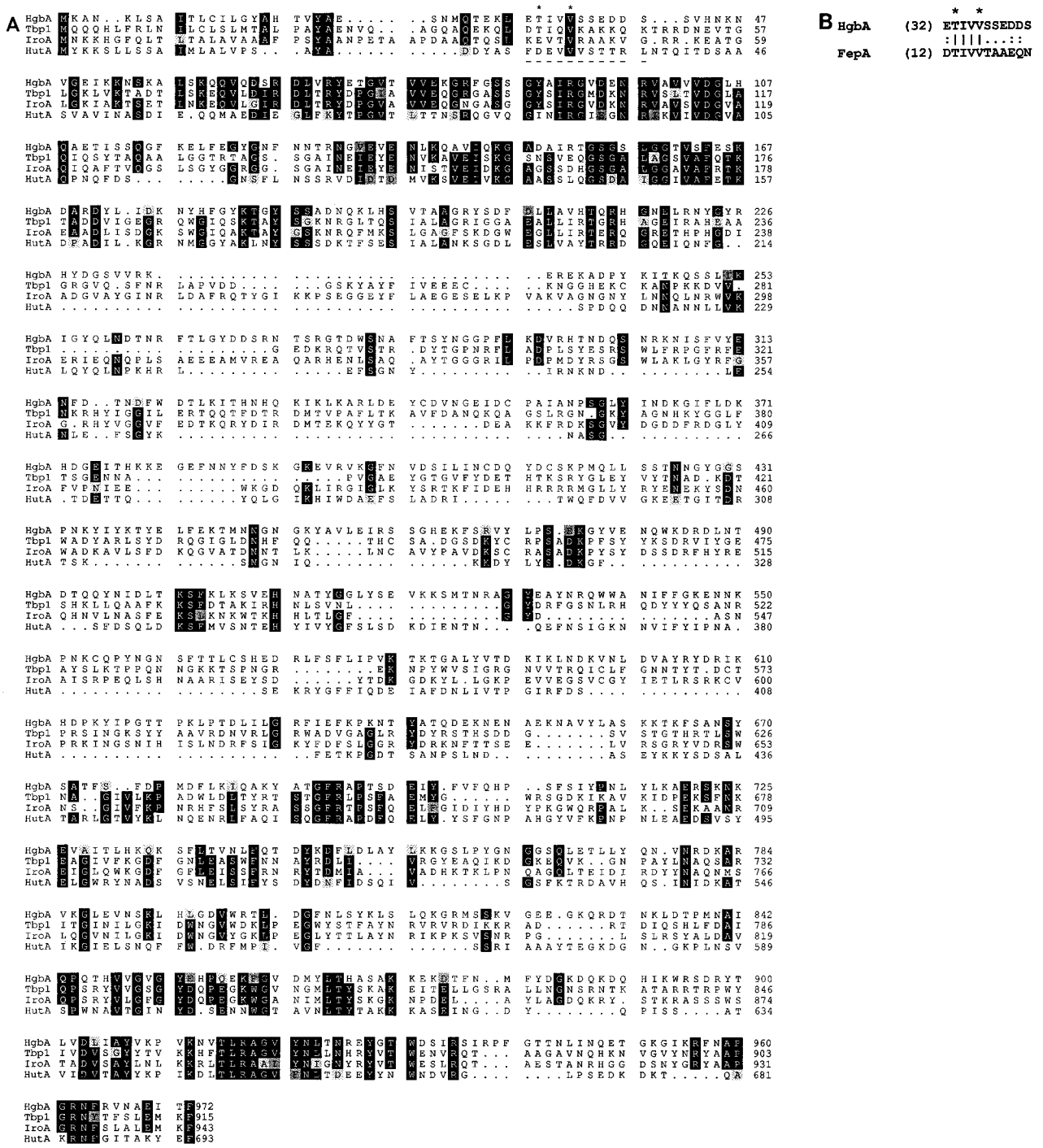

FIG. 5. Homology between HgbA and TonB-dependent outer membrane proteins. (A) A comparison of the amino acid sequences of HgbA and the three proteins most similar to it found in GenBank is shown. HgbA, Hgb-binding protein from *H. ducreyi* 35000 (this study); Tbp1, transferrin-binding protein from gonococcal strain FA19 (3); Lbp1 (IroA), lactoferrin-binding protein from meningococcal strain BNCV (22); HutA, heme receptor from *V. cholerae* CA401 (9). The alignment was generated with the default parameters in the Pileup program (5) and by manual alignment of the signal peptide and TonB boxes. The 11 residues of the putative TonB boxes are underlined. The asterisks over the T and V residues indicate the two most conserved positions within the TonB boxes (3). The reversed-face letters indicate highly conserved residues; the shaded letters indicate moderately conserved substitutions. (B). Homology of the TonB boxes of *E. coli* FepA (15) and *H. ducreyi* HgbA. Vertical lines, identical residues; colons, conserved substitutions; dots, semiconserved substitutions.

(30), but an alternative sequence VYA was present. This signal peptidase I cleavage site is similar to those proposed for other related receptor proteins (Fig. 5) (3) and is identical to that of meningococcal Tbp1 from strain B16B6 (14). Cleavage at this site would yield a mature deduced protein of 108,572 Da, which is in agreement with the observed mobility on SDS-PAGE gels. The calculated pI of HgbA is 9.9 and is consistent with the basic nature of the related receptor proteins (see below).

TABLE 1. Similarity and identity of proteins related to HgbA

Protein	Bacterial species	% Similar	% Identical	Reference
HutA	*V. cholerae*	55	31	9
Tbp1	*N. gonorrhoeae* and *N. meningitidis*	47–50	25–27	3, 14
Lbp1 (IroA)	*N. gonorrhoeae* and *N. meningitidis*	48–50	25–26	4, 22
HemR	*Yersinia pestis*	48	26	26
FepA	*E. coli*	47	24	15

Homology to other TonB-dependent proteins. The results of a BLASTX search (2a) of GenBank revealed that the HgbA protein was highly related to a group of TonB-dependent receptor proteins found in other mucosal pathogens (Table 1) (4). The most similar protein was HutA (9), a 74-kDa heme receptor from *Vibrio cholerae*, followed closely by the 100-kDa transferrin receptors (3, 14) and lactoferrin receptor Lbp1 (IroA) (22) from *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Somewhat less but still significant homology was observed with *E. coli* FepA (15) and *Yersinia enterocolitica* HemR (26). A comparison of HgbA with selected members of this family is shown in Fig. 5A; for reasons of brevity, only three highly related proteins are compared.

FepA and certain other related Ton B-dependent receptors from *E. coli* have been shown experimentally to be dependent upon TonB for function (7, 15). Tbp1, IroA, and HutA have been inferred to be TonB dependent on the basis of homology to FepA and others. Several interspersed regions are conserved throughout the TonB-dependent outer membrane protein receptors (3, 18). One region in particular, termed the TonB box, located near the N terminus is highly conserved in *E. coli* and is important in TonB function (7). The putative TonB boxes of HgbA, Tbp1 (3), IroA (22), and HutA (9) were aligned and underlined. Significant sequence diversity existed among the TonB boxes of these four proteins. However, when the TonB box of HgbA was compared with the FepA TonB box (Fig. 5B), the comparison revealed that 10 of 11 residues were similar and that 2 invariant residues were present in HgbA (3). Other regions of homology within the TonB-dependent family of proteins contain certain (invariant) residues (3, 18). HgbA contains 12 of 13 invariant residues. Therefore, on the basis of homology to FepA, we suggest that HgbA may be a TonB-dependent receptor.

Construction of an isogenic *hgbA* mutant. The strategy of allelic replacement of *hgbA* was used to make an isogenic mutant. *H. ducreyi* 35000 was electroporated with linearized DNAs from each of the three mutagenized plasmids shown in Fig. 2. Transformants were obtained with pUNCH 580 and pUNCH 581 but were not obtained with pUNCH 582. Chloramphenicol-resistant *H. ducreyi* transformants were screened for the inability to grow on Hgb agar plates and by SDS-PAGE for lack of HgbA (data not shown). One *H. ducreyi hgbA* isogenic mutant obtained by electroporation with pUNCH 580 was termed FX504 and was extensively characterized. Another *hgbA* mutant obtained by electroporation with pUNCH 581 and termed FX507 had a phenotype identical with that of FX504 (data not shown).

Southern blot analysis of mutant FX504. To confirm that FX504 arose as a simple allelic exchange event between pUNCH 580 and the chromosome of *H. ducreyi* 35000, we performed Southern blots of *Eco*RI digests of strain 35000 and FX504 (Fig. 6). The blots were probed with pUNCH 579, which contains the *hgbA* gene (Fig. 6A), or pNC 40, which contains the CAT cassette (Fig. 6B). The insert of pUNCH 579 hybridized to itself, a comigrating 8.5-kb *Eco*RI fragment in *H. ducreyi* 35000, and the two halves of the mutagenized 8.5-kb *Eco*RI fragment in both pUNCH 580 and FX504 (the CAT cassette introduces an additional 1.6 kb of DNA containing an additional *Eco*RI site into the middle of the *Eco*RI fragment from pUNCH 580). The CAT cassette hybridized only to the two halves of the mutagenized 8.5-kb *Eco*RI fragment in both pUNCH 580 and FX504. The hybridization with a 3-kb fragment in both plasmid digests shown in both panels of Fig. 6 was due to the presence of plasmid sequences in the DNA used to make the probes. These data indicated that FX504 was the result of a double crossover gene replacement event between pUNCH 580 and *H. ducreyi* 35000.

Characterization of isogenic mutant FX504. The *hgbA* mutant FX504 was further characterized by performing Western blots and dot blots. FX504 expressed no detectable HgbA by Western blot analysis and no Hgb binding activity in dot blots (Fig. 3A and B, respectively; compare lanes 1 and 2).

The ability of the *hgbA* mutant FX504 to grow on human Hgb was tested by preparing GCB-I plates containing various concentrations of Hgb. Single colonies were streaked for isolation onto plates to obtain data (Fig. 7). Mutant FX504 could not grow on plates containing 100 μg of human Hgb per ml, whereas the wild-type 35000 grew well (Fig. 7). Both strains grew on GCB-I plates with 50 μg of heme per ml (positive control), and neither strain grew on GCB-I plates without heme or Hgb (negative control) (data not shown). The minimum amount of Hgb which could support the growth of parent *H. ducreyi* 35000 varied from 50 to 100 μg/ml. Depending on the lot of Hgb agar, FX504 grew in some experiments on 500 or 1,000 μg of Hgb per ml, but in all experiments, the wild-type 35000 always exhibited an at least 10-fold lower requirement for human Hgb than FX504 (data not shown).

The ability of the *hgbA* mutant FX504 to grow on human blood agar plates was also tested. Initial subculture of a single

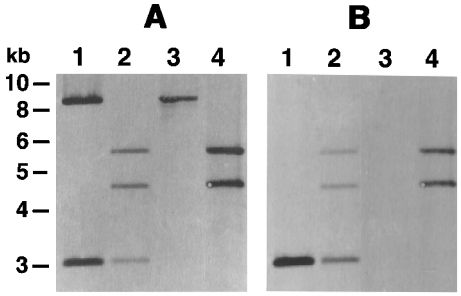

FIG. 6. Southern blotting of *H. ducreyi* 35000 and *hgbA* mutant FX504. Plasmids pUNCH 579 and pUNCH 580 and chromosomal DNA from *H. ducreyi* 35000 and *hgbA* mutant FX504 were each digested with *Eco*RI and subjected to Southern blotting. The membrane was first probed with random primed pUNCH 579 (A), stripped, and then reprobed with random primed pNC 40 (containing the CAT cassette) (B). Lane 1, plasmid pUNCH 579; lane 2, plasmid pUNCH 580; lane 3, chromosomal DNA from *H. ducreyi* 35000; lane 4, chromosomal DNA from *hgbA* mutant FX504.

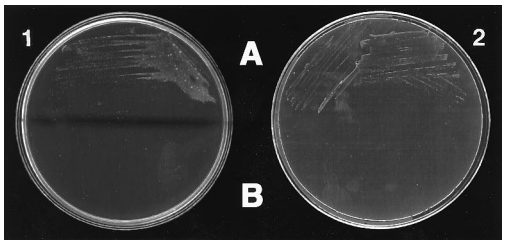

FIG. 7. Ability of *H. ducreyi* 35000 and *hgbA* mutant FX504 to use human Hgb as a source of heme or to grow on human blood agar plates. *H. ducreyi* 35000 (A) and *hgbA* mutant FX504 (B) were streaked onto a GCB-I agar plate (plate 1) containing 100 μg of human hemoglobin per ml and were streaked onto a GCB-I agar plate (plate 2) containing 1% washed human erythrocytes and incubated at 35°C for 48 to 72 h. See the text for details. Similar results were seen in at least two other experiments.

colony by the streaking of FX504 onto 5% human blood agar resulted in tiny colonies which appeared to be strongly beta-hemolytic, considering the sparse growth (data not shown). The parent *H. ducreyi* 35000 grew well but was minimally beta-hemolytic. A subculture from growth on the first blood plate to a second blood agar plate resulted in no growth for FX504 and continued normal growth of *H. ducreyi* 35000 (Fig. 7B).

It has previously been shown that animal Hgbs can support the growth of *H. ducreyi* as well as compete with human Hgb for binding to *H. ducreyi* (6). The ability of animal Hgbs to support the growth of FX504 was further tested. Baboon, pig, sheep, horse, bovine, dog, rabbit, or mouse Hgb could not support the growth of FX504, while all supported the growth of *H. ducreyi* 35000 (data not shown).

DISCUSSION

Purification of HgbA (6) led to the development of a degenerate oligonucleotide and an N-terminal peptide serum, which were used to clone and characterize *hgbA*. Even though the deduced oligonucleotide HgbA.01 was 64-fold degenerate, it hybridized strongly in Southern blots and was used successfully in sequencing reactions to obtain preliminary DNA sequences. The N-terminal anti-peptide serum helped to prove that we had cloned *hgbA*, established the orientation and localization of *hgbA*, and confirmed that the *hgbA* mutants did not produce HgbA.

Cloning of *hgbA* was readily performed by standard methods. We did notice, however, that clones expressing the full-length HgbA product grew more slowly and were more likely to undergo lysis and that pUNCH 579 was somewhat unstable.

In addition to *E. coli* DH5αMCR, three other strains of *E. coli* harboring pUNCH 579 bound Hgb (data not shown), thus establishing HgbA as an Hgb receptor. The cellular location of HgbA in *E. coli* is uncertain. We were unable to affinity purify the protein by the same technique that we used for *H. ducreyi* (6). The binding seen in the whole-cell dot blots of *E. coli* expressing *hgbA* could have been due to the outer membrane localization of HgbA. Since *E. coli* clones expressing *hgbA* lysed more readily, lysis of the cells during the assay could have resulted in the exposure of cytoplasmic or periplasmic HgbA and subsequent binding activity. Regardless, the binding demonstrated by *E. coli* expressing *hgbA* was specific, since it could be inhibited by unlabeled Hgb (Fig. 3C).

The DNA sequence of *hgbA* was typical of those of other members of this family of outer membrane receptors. DNA from *Haemophilus* spp. is AT rich, and the *hgbA* sequence shown contains 65% AT nucleotides. Since −10, −35, and Fur box sequences are also high in AT nucleotides, assigning functional significance to AT-rich DNA is problematic. Thus, these designations should be considered tentative until proven experimentally. The level of HgbA in *E. coli* DH5αMCR was not regulated by the chelator 2,2-dipyridyl, possibly because of a copy effect or lack of heterologous repression.

The similarity of HgbA and other established TonB-dependent receptors suggests that HgbA is a receptor. Many receptors of this class are involved in iron acquisition and have been shown to be transport proteins for their ligands (such as FepA, HemR, and HutA). Other receptors such as Tbp1, alone or in conjunction with other proteins, remove iron from the host iron-containing protein and transport iron across the outer membrane. It is possible that HgbA could remove the heme from Hgb and transport heme across the outer membrane. We tested whether the *hgbA* gene product could complement the growth of *hemA E. coli* RK1065 (11). However, the results were uninterpretable, since a control for leakage of the periplasmic enzyme RNase I (33) by the recombinant *hemA* strain expressing HgbA was strongly positive (data not shown). Thus, if RNase I could leak out, heme could leak in, invalidating any complementation experiments.

Recently, the gene encoding the TonB protein from *Haemophilus influenzae* was cloned and sequenced (10). A functional TonB protein is required in *H. influenzae* for heme utilization (10). These data, taken together with the TonB box homology present in HgbA, make it likely that a TonB protein exists not only in *H. influenzae* but in *H. ducreyi* also. If an *H. ducreyi* TonB homolog gene is cloned, the TonB dependence of HgbA can be ascertained in its homologous system, where HgbA does not cause lysis or leakage.

Other members of this receptor family are known to be regulated by the negative transcriptional repressor Fur (20). Under high-iron growth conditions, Fur complexes with iron and binds to the Fur box of iron-regulated genes, blocking transcription. Under low-iron growth conditions, Fur is not complexed with iron and disassociates from the promoters and the genes are derepressed. In contrast, it is reported that low-heme conditions, not low-iron conditions, enhanced the expression of HgbA in *H. ducreyi* (6). If heme is the major source of intracellular iron in *H. ducreyi*, Fur could be responsible for the repression of *hgbA* expression under high-heme conditions.

A comparison of the HgbA sequence with the individual peptide sequences of Tbp1, Lbp1 (IroA), HutA, HemR, and FepA yielded significant similarities (Table 1). Even more revealing was the comparison of the four proteins in Fig. 5A. A region of striking dissimilarity was present in the central regions of these four proteins (residues 220 to 670 for HgbA; Fig. 5A). Tbp1, Lbp1 (IroA), and HgbA (which bind protein ligands) contain certain domains absent from the heme receptor HutA (which binds heme). Interestingly, Tbp1, Lbp1, and HgbA contain divergent sequences in this domain. On the basis of these observations, it is tempting to speculate that this region of Tbp1, Lbp1, and HgbA contains the ligand binding domain and that the diversity found in this region specifies the ligand bound.

Mutagenesis of *hgbA* in *H. ducreyi* was performed by the technique of electroporation (8). *H. ducreyi* mutant FX504 did not express *hgbA* by Western blotting, bind human Hgb, or grow on human Hgb or human blood plates. The initial sparse growth of FX504 on human blood plates might have been due to residual heme stores or possibly some heme leakage from the erythrocytes, since these colonies were beta-hemolytic. The strong beta-hemolysis observed with FX504 on this initial subculture may indicate that the hemolysin of *H. ducreyi* is heme or iron regulated. The inability to obtain *H. ducreyi* transfor-

mants with pUNCH 582 could have been due to the lethality of this mutation, insufficient flanking DNA for recombination, or other technical reasons.

It has been shown that various animal Hgbs can serve as a source of heme for *H. ducreyi* in vitro. All tested animal Hgbs supported the growth of wild-type strain 35000, but none supported that of HgbA isogenic mutant FX504. Thus, the role of the Hgb receptor in the establishment and pathogenesis of infection can be directly tested in animal models.

ACKNOWLEDGMENTS

We thank Cynthia Cornelissen, P. Frederick Sparling, Dan Morton, Terrance Stull, and Phillip Klebba for their helpful comments; members of the Sparling laboratory for critiquing the manuscript; and Annice Roundtree for her expert technical assistance.

The work presented here was supported by a developmental grant from the North Carolina Sexually Transmitted Disease Infections Research Center, University of North Carolina, Chapel Hill, N.C., to C.E. and Public Health Service grants AI26837 and AI31496 to P. Frederick Sparling.

REFERENCES

1. **Albritton, W. L.** 1989. Biology of *Haemophilus ducreyi*. Microbiol. Rev. **53:** 377–389.
2. **Albritton, W. L., I. W. Macklean, P. D. Bertram, and A. R. Ronald.** 1981. Haemin requirements in *Haemophilus* with special reference to *H. ducreyi*, p. 75–82. *In* M. Kilin, W. Fredericksen, and E. L. Biberstein (ed.), *Haemophilus*, *Pasteurella* and *Actinobacillus*. Academic Press, New York.

2a. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.

3. **Cornelissen, C. N., G. D. Biswas, J. Tsai, D. K. Paruchuri, S. A. Thompson, and P. F. Sparling.** 1992. Gonococcal transferrin-binding protein 1 is required for transferrin utilization and is homologous to TonB-dependent outer membrane receptors. J. Bacteriol. **174:**5788–5797.
4. **Cornelissen, C. N., and P. F. Sparling.** 1994. Iron piracy: iron acquisition from transferrin by bacterial pathogens. Mol. Microbiol. **14:**843–850.
5. **Deveraux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
6. **Elkins, C.** 1995. Identification and purification of a conserved heme-regulated hemoglobin-binding outer membrane protein from *Haemophilus ducreyi*. Infect. Immun. **63:**1241–1245.
7. **Gudmundsdottir, A., P. E. Bell, M. D. Lundrigan, C. Bradbeer, and R. J. Kadner.** 1989. Point mutations in a conserved region (TonB box) of *Escherichia coli* outer membrane protein BtuB affect vitamin B_{12} transport. J. Bacteriol. **171:**6526–6533.
8. **Hansen, E. J., J. L. Latimer, S. E. Thomas, M. Helminen, W. L. Albritton, and J. D. Radolf.** 1992. Use of electroporation to construct isogenic mutants of *Haemophilus ducreyi*. J. Bacteriol. **174:**5442–5449.
9. **Henderson, D. P., and S. M. Payne.** 1994. Characterization of the *Vibrio cholerae* outer membrane heme transport protein HutA: sequence of the gene, regulation of expression, and homology to the family of TonB-dependent proteins. J. Bacteriol. **176:**3269–3277.
10. **Jarosik, G. P., J. D. Sanders, L. D. Cope, U. Muller-Eberhard, and E. J. Hansen.** 1994. A functional TonB gene is required for both utilization of heme and virulence expression by *Haemophilus influenzae* type B. Infect. Immun. **62:**2470–2477.
11. **Kadner, R.** Unpublished data.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
13. **Lee, B. C.** 1991. Iron sources for *Haemophilus ducreyi*. J. Med. Microbiol. **34:**317–322.
14. **Legrain, M., V. Mazarin, S. W. Irwin, B. Bouchon, M. J. Quentin-Millet, E. Jacobs, and A. B. Schryvers.** 1993. Cloning and characterization of Neisseria meningitidis genes encoding the transferrin-binding proteins Tbp1 and Tbp2. Gene **130:**73–80.
15. **Lundrigan, M. D., and R. J. Kadner.** 1986. Nucleotide sequence of the gene for the ferrienterochelin receptor FepA in Escherichia coli. Homology among outer membrane receptors that interact with TonB. J. Biol. Chem. **261:**10797–10801.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Morse, S. A.** 1989. Chancroid and *Haemophilus ducreyi*. Clin. Microbiol. Rev. **2:**137–157.
18. **Nau, C. D., and J. Konisky.** 1989. Evolutionary relationship between the TonB-dependent outer membrane transport proteins: nucleotide and amino acid sequences of the *Escherichia coli* colicin I receptor gene. J. Bacteriol. **171:**1041–1047.
19. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Biochem. **50:** 715–731.
20. **Otto, B. R., A. M. J. J. V.-v. Vught, and D. M. MacLaren.** 1992. Transferrins and heme-compounds as iron sources for pathogenic bacteria. Crit. Rev. Microbiol. **18:**217–233.
21. **Palmer, K. L., S. Grass, and R. S. Munson.** 1994. Identification of a hemolytic activity elaborated by *Haemophilus ducreyi.* Infect. Immun. **62:**3041–3043.
22. **Pettersson, A., P. van der Ley, J. T. Poolman, and J. Tommassen.** 1993. Molecular characterization of the 98-kilodalton iron-regulated outer membrane protein of *Neisseria meningitidis*. Infect. Immun. **61:**4724–4733.
23. **Plummer, F. A., J. N. Simonsen, D. W. Cameron, J. O. Ndinya-Achola, J. K. Kreiss, M. N. Gakinya, P. Waiyaki, M. Cheang, P. Piot, A. R. Ronald, and E. N. Ngugi.** 1991. Cofactors in male-female sexual transmission of human immunodeficiency virus type 1. J. Infect. Dis. **163:**233–239.
24. **Ronald, A. R., and W. Albritton.** 1990. Chancroid and *Haemophilus ducreyi*, p. 263–272. *In* K. K. Holmes, P.-A. Mardh, P. F. Sparling, and P. J. Wiesner (ed.), Sexually transmitted diseases, 2nd ed. McGraw-Hill, New York.
25. **Seifert, H. S., E. Y. Chen, M. So, and F. Heffron.** 1986. Shuttle mutagenesis: a method of transposon mutagenesis for *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **83:**735–739.
26. **Stojiljkovic, I., and K. Hantke.** 1992. Hemin uptake system of *Yersinia enterocolitica*: similarities with other TonB-dependent systems in gram-negative bacteria. EMBO J. **11:**4359–4367.
27. **Struyve, M., M. Moons, and J. Tommassen.** 1991. Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. J. Mol. Biol. **218:**141–148.
28. **Thomas, C. E., N. H. Carbonetti, and P. F. Sparling.** Unpublished data.
29. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
30. **von Heijne, G.** 1990. The signal peptide. J. Membr. Biol. **115:**195–201.
31. **Wasserheit, J. N.** 1991. Interrelationships between human immunodeficiency virus infection and other sexually transmitted diseases. Sex. Transm. Dis. **19:**61–77.
32. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42:**45–66.
33. **Young, K., and L. L. Silver.** 1991. Leakage of periplasmic enzymes from *envA1* strains of *Escherichia coli*. J. Bacteriol. **173:**3609–3614.