INFECTION AND IMMUNITY, May 1996, p. 1724–1735 Vol. 64, No. 5
0019-9567/96/$04.00+0

A Hemoglobin-Binding Outer Membrane Protein Is Involved in Virulence Expression by *Haemophilus ducreyi* in an Animal Model

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Received 22 August 1995/Returned for modification 31 October 1995/Accepted 6 February 1996

Haemophilus ducreyi **exhibits a requirement for exogenously supplied heme for aerobic growth in vitro. Nine of ten wild-type isolates of *H. ducreyi* were shown to contain a readily detectable hemoglobin-binding activity. Spontaneous hemoglobin-binding-negative mutants of two of these wild-type isolates lost the ability to express an outer membrane protein with an apparent molecular mass of approximately 100 kDa. Similarly, the single wild-type isolate that lacked the ability to bind hemoglobin also appeared to lack expression of this same 100-kDa protein. A monoclonal antibody (5A9) to this 100-kDa protein was used to identify a recombinant clone which possessed an *H. ducreyi* chromosomal fragment containing the gene encoding the 100-kDa protein; this protein was designated hemoglobin utilization protein A (HupA). Nucleotide sequence analysis of the *hupA* gene revealed that the predicted protein, with a calculated molecular mass of 108 kDa, was similar to TonB-dependent outer membrane proteins of other bacteria. Increasing the concentration of heme in the growth medium resulted in decreased expression of the HupA protein. Mutant analysis was used to prove that the HupA protein was essential for the utilization by *H. ducreyi* of both hemoglobin and hemoglobin-haptoglobin as sources of heme in vitro. In addition, it was found that an isogenic *hupA* mutant was less virulent than the wild-type parent strain in the temperature-dependent rabbit model for dermal lesion production by *H. ducreyi*.**

Haemophilus ducreyi, the causative agent of chancroid, is the subject of increased research interest because of the demonstrated association between genital ulcers and increased transmission of the human immunodeficiency virus (7, 22, 37, 50–52). This sexually transmitted pathogen is especially noted for its fastidious nature (1, 46, 58), such that recovery of *H. ducreyi* from genital ulcers is difficult even for very experienced clinical laboratories (16, 39, 47, 58). The demanding nature of this organism's metabolism, coupled with its notorious tendency to autoagglutinate (1, 46), has impeded efforts to elucidate the basic metabolic and physiologic characteristics of this bacterium.

One of the more interesting phenotypic attributes of this sexually transmitted pathogen is its requirement for exogenously supplied heme for aerobic growth in vitro. Although the requirement of *H. ducreyi* for heme has been known for many years (1, 2, 24), surprisingly little attention has been paid to understanding how this bacterium satisfies this unusual nutritional requirement, especially while growing in its human host. In fact, it has been reported that the level of free heme necessary for growth of *H. ducreyi* in vitro (2, 24) is an order of magnitude greater than that for *Haemophilus influenzae*, the classic heme-requiring pathogen (19). In this context, it also should be noted that nothing is known about the metabolic and physiologic factors of *H. ducreyi* which contribute to its ability to proliferate in the dermis. The heme source(s) actually utilized by *H. ducreyi* growing in vivo is open to speculation, but the production of a hemolysin by this pathogen (49) suggests that *H. ducreyi* is able to effect the release of hemoglobin from erythrocytes.

It has been reported that *H. ducreyi* can use a number of heme sources for growth in vitro, including free heme, hemoglobin, and hemoglobin complexed with the serum protein haptoglobin (40). The goal of the present study was to identify one or more *H. ducreyi* gene products involved in heme acquisition and to determine whether these *H. ducreyi* proteins played any role in virulence expression by this pathogen in an animal model. To this end, we identified a 108-kDa outer membrane protein that is (i) essential to the ability of *H. ducreyi* to utilize both hemoglobin and hemoglobin-haptoglobin as sources of heme for growth and (ii) required for expression of full virulence by this pathogen in an animal model.

(A preliminary report of this work was presented previously [62a].)

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1 together with their geographic sources of isolation (for *H. ducreyi* strains) and their relevant phenotypes or genotypes. *H. ducreyi* strains were routinely cultivated on chocolate agar plates containing 1% (vol/vol) IsoVitalex (BBL Microbiological Systems, Cockeysville, Md.) as described previously (54). Other media for growth of *H. ducreyi* included a clear medium described by Lee (40) which consisted of GC agar base (BBL) supplemented with 0.1% (wt/vol) glucose, 0.01% (wt/vol) L-glutamine, 0.025% (wt/vol) L-cysteine–HCl, and a heme source: equine hemin (Sigma Chemical Co., St. Louis, Mo.) at 12.5, 25, 50, or 100 μg/ml; human hemoglobin (Sigma) at 250 μg/ml; or human hemoglobin-haptoglobin at 16 μg/ml. Human haptoglobin (Calbiochem, La Jolla, Calif.) was loaded with human hemoglobin as described previously (6). *Escherichia coli* strains were grown in LB medium (59). Antimicrobial agent supplementation for certain experiments with *H. ducreyi* included chloramphenicol at a final concentration of 2 μg/ml or kanamycin at 30 μg/ml. For recombinant *E. coli* strains, antimicrobial agent supplementation included ampicillin at a final concentration of 100 μg/ml (for pUC19-derived plasmids), kanamycin at 50 μg/ml (for pBK-CMV-derived plasmids), or chloramphenicol at 30 μg/ml (for plasmids containing the *cat* cartridge).

For measurement of the growth rate of *H. ducreyi* strains, cells were grown on

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TABLE 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or description	Source or reference
H. ducreyi strains		
35000	Wild-type strain isolated in Winnipeg, Manitoba, Canada	25, 54
35000.21	Isogenic *hupA* mutant, obtained by electroporating 35000 with linearized pSL706; this mutant does not express the 108-kDa HupA protein and cannot utilize hemoglobin as a source of heme	This study
35000-102	35000 with a *cat* cartridge inserted into a gene encoding a 49-kDa cell envelope antigen; this strain is as virulent as 35000 in the rabbit model	27
Cha-1	Wild-type strain isolated in Dallas, Tex.	28
Cha-1.1	Spontaneous mutant unable to express the 108-kDa HupA protein; this mutant cannot utilize hemoglobin as a source of heme	This study
STD101	Wild-type strain isolated in Dallas, Tex.	This study
Hd13	Wild-type strain isolated in Singapore	56
1151	Wild-type strain isolated in the Gambia	Alan Ronald
E1673	Wild-type strain isolated in Sweden	3
041	Wild-type strain isolated in Sweden	Allan Ronald
1145	Wild-type strain isolated in Amsterdam, The Netherlands	Allan Ronald
1352	Wild-type strain isolated in Kenya	Allan Ronald
512	Wild-type strain isolated in Thailand; this strain does not express the HupA protein and is unable to utilize hemoglobin as a source of heme	Allan Ronald
E. coli strains		
XL0LR	Host strain used for excising the recombinant pBK-CMV phagemids from Lambda ZAP Express bacteriophages	Stratagene
DH5α	Host strain for the pUC19-based genomic library	59
HB101	Host strain used for selecting the mutated *hupA* gene containing the *cat* cartridge	59
Plasmids		
pBK-CMV	Phagemid excised from Lambda ZAP Express cloning vector	Stratagene
pSL705	pBK-CMV with the 3.2-kb *H. ducreyi* insert containing nearly all of the *hupA* gene	This study
pLS706	pSL705 containing a *cat* cartridge inserted into the *Pfl*MI site within the *hupA* gene	This study
pUC19	Cloning vector, Amp^r	
pSL707	pUC19 with a 2.7-kb fragment of *H. ducreyi* chromosomal DNA containing the 3′ end of the *hupA* ORF	This study

the clear medium described above lacking a heme source for 36 to 40 h under anaerobic conditions at 33°C (to eliminate intracellular heme reserves). These cells were suspended in sterile phosphate-buffered saline (pH 7.4) to an optical density at 600 nm of 0.5 in a 50-ml conical centrifuge tube. After a wait of 30 s to allow large clumps to settle, the upper half of the suspension was transferred to another tube and 3-ml portions of this suspension were used to inoculate 500-ml sidearm flasks containing 150 ml of broth culture medium. This culture medium consisted of filtration-sterilized brain heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 1% (vol/vol) IsoVitalex, 5% (vol/vol) fetal bovine serum, and heme at a final concentration of either 5 or 50 μg/ml. Growth was monitored by measuring the change in optical density at 600 nm over time. Growth experiments were repeated three times.

Recombinant DNA techniques. Standard recombinant DNA techniques including restriction enzyme digestions, ligation reactions, agarose gel electrophoresis, and plasmid purification were performed as previously described (44, 59). The *cat* cartridge encoding chloramphenicol acetyltransferase was derived from the mutated plasmid pUCΔEcat, which was kindly provided by Bruce A. Green. Screening of recombinant *E. coli* clones for reactivity with the 700-bp *Bgl*II-*Eco*RI fragment from pSL705 was accomplished by previously published methods (44, 59), using the Random Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, Ind.) to radiolabel this DNA fragment.

Construction of *H. ducreyi* genomic libraries. Chromosomal DNA from *H. ducreyi* 35000 was partially digested with *Sau*3A1, and fragments ranging in size from 3 to 8 kb, purified in sucrose density gradients, were used to construct a genomic library in the Lambda ZAP Express vector (Stratagene Cloning Systems, La Jolla, Calif.), using the protocol provided by the manufacturer. This genomic library contained 10^7 recombinant PFU. Excision of the desired recombinant DNA fragment containing the gene encoding the 100-kDa protein was accomplished by the protocol provided by the manufacturer and yielded the recombinant plasmid pSL705. A second genomic library containing 40,000 recombinant clones was constructed by ligating *Sau*3AI fragments of *H. ducreyi* 35000 chromosomal DNA into the *Bam*HI site in the plasmid vector pUC19. The recombinant plasmid pSL707 was derived from the latter genomic library.

Transposon-mediated mutagenesis system. The wild-type Cha-1 and 35000 strains were subjected to transposon-mediated mutagenesis by using the pMS1/Tn*1545*-Δ*3*-based system described previously (62). Selection of transformants containing transposon insertions involved the use of GC-heme agar containing kanamycin (30 μg/ml).

Hemoglobin- and hemoglobin-haptoglobin-binding assays. *H. ducreyi* strains were grown on GC agar media containing heme at final concentrations of 12.5, 50, and 100 μg/ml. The resultant colony paste was transferred in duplicate by applicator stick to Whatman no. 40 filter paper, dried for 1 h at 37°C, and blocked for 1 h in phosphate-buffered saline (pH 7.3) containing 1% (wt/vol) bovine serum albumin and 0.05% (vol/vol) Tween-20 (PBS-BSA-T). The filter paper was then incubated overnight at 4°C in PBS-BSA-T containing 2×10^6 cpm of human hemoglobin or hemoglobin-haptoglobin that had been radioiodinated to a specific activity of 10^7 cpm/μg. After three washes with PBS-BSA-T, the filter was dried and processed for autoradiography.

SDS-PAGE and Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was accomplished as described previously (23) using *H. ducreyi* whole-cell lysates (27), cell envelope preparations (27), or the Sarkosyl-insoluble fraction from cell envelopes (29) as the source of protein. Western blot (immunoblot) analysis of the reactivity of the 100-kDa protein with polyclonal mouse antiserum and with monoclonal antibody (MAb) 5A9 was accomplished as described previously (23). MAb 3F12 is specific for the major outer membrane protein of *H. ducreyi* (61) and was included in a Western blot experiment to standardize antigen loads.

Production of a MAb reactive with the 100-kDa hemoglobin-binding protein. Cell envelopes were prepared from *H. ducreyi* 35000 as described previously (28). Proteins present in these envelopes were resolved by SDS-PAGE, and the 100-kDa protein band was eluted from gel slices by using an Elutrap electroelution system (Schleicher & Schuell, Keene, N.H.). The concentrated protein suspension was precipitated by adding 1/10 volume of a solution containing 100% (wt/vol) trichloroacetic acid and sodium deoxycholate (0.4 mg/ml) and incubating the suspension in crushed ice for 30 min. The precipitate was collected by centrifugation at $16,000 \times g$ for 30 min and washed once with ice-cold acetone. After centrifugation, the pellet was suspended in PBS containing 0.05% (wt/vol) SDS. Spleen cells from mice immunized with this protein preparation were used in our standard hybridoma fusion protocol (57). A lymphocyte hybridoma, designated 5A9, was identified which secreted a MAb that bound the 100-kDa protein in Western blot analysis. In addition, sera obtained from these same mice at the time of the removal of their spleens were shown, by Western blot analysis, to contain polyclonal antibodies to the 100-kDa protein.

N-terminal amino acid sequence analysis. Cell envelopes of the wild-type strain 35000 were solubilized, and the proteins were resolved by SDS-PAGE as described above. The 100-kDa protein band was excised from these gels and then subjected to SDS-PAGE by the method of Hunkapiller et al. (35). The gel-resolved protein band was transferred to a polyvinylidene difluoride membrane

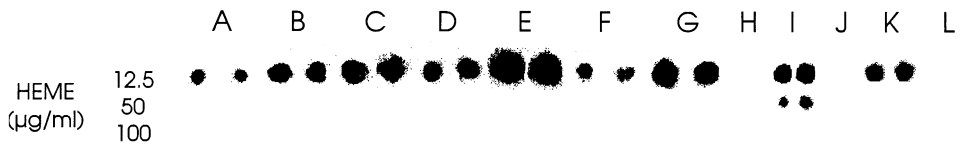

FIG. 1. Autoradiograph of binding of radioiodinated hemoglobin by wild-type and mutant strains of *H. ducreyi*. Cell paste from *H. ducreyi* strains grown overnight on GC agar-based media containing heme at final concentrations of 12.5, 50, and 100 μg/ml was transferred in duplicate onto filter paper and incubated with radiolabeled human hemoglobin. Columns: A, 1151; B, E1673; C, 041; D, 1145; E, Hd13; F, STD101; G, 1352; H, 512; I, Cha-1; J, Cha-1.1; K, 35000; L, 35000.21.

by the method of Matsudaira (45) and subjected to N-terminal amino acid analysis as described previously (26).

Nucleotide sequence analysis. Both strands of the 3.2-kb *H. ducreyi* insert in the recombinant plasmid pSL705 were sequenced in their entirety, as was the 5′ end of the *H. ducreyi* DNA insert in pSL707. DNA sequence information was analyzed through the National Center for Biotechnology Information using the BLAST network service to search GenBank (4) and by using programs from the University of Wisconsin Genetics Computer Group software sequence analysis package (14).

Construction of an isogenic mutant unable to express the 100-kDa (HupA) protein. The recombinant plasmid pSL705 was digested to completion with *Pfl*MI, which cuts once within the structural gene encoding the HupA protein. The linearized plasmid was treated with Klenow fragment (New England Biolabs, Beverly, Mass.) to create blunt ends and with calf alkaline phosphatase (Boehringer Mannheim). The *cat* cartridge containing *Eco*RI ends was also treated with Klenow fragment to create blunt ends and was ligated into this site. The ligation reaction mixture was used to transform *E. coli* HB101, which was then plated onto LB agar containing chloramphenicol to select the desired recombinants with a *cat* cartridge inserted into the *hupA* structural gene. Plasmid pSL706 was obtained in this manner and shown to contain the *cat* cartridge at the desired site within the *hupA* gene. Plasmid pSL706 was linearized by digestion with *Pst*I, which cuts this plasmid once within the vector backbone. This preparation was desalted by precipitation with ethanol and then suspended in water. A 6-μg quantity of this DNA was used to electroporate the wild-type *H. ducreyi* strain 35000 as described previously (27), and transformants were selected on GC-heme agar containing chloramphenicol.

Southern blot analysis. Chromosomal DNA purified from wild-type and mutant *H. ducreyi* strains was digested to completion with appropriate restriction enzymes and probed in Southern blot analysis as described by Sambrook et al. (59). The DNA probes used in these experiments included the 1.4-kb *cat* gene from pUCΔEcat and a 1.6-kb *Afl*II-*Pfl*MI fragment from within the *hupA* open reading frame.

Virulence testing. Male New Zealand White rabbits (2.7 to 3.2 kg) were obtained from commercial sources and housed in rooms with temperatures controlled at 15 to 17°C (54). At least 5 days before bacterial inoculation, the dorsal hair of each rabbit was shaved with an animal-grooming clipper (Model A-5; Oster Professional Products, Milwaukee, Wis.). The rabbit backs were shaved every other day until infection with *H. ducreyi*, after which they were shaved daily.

The *H. ducreyi* strains used for inoculation of animals were grown for 16 to 18 h and were harvested by scraping bacterial growth from chocolate agar plates into sterile PBS. Cells were washed once in PBS by centrifugation and resuspension, and serial 10-fold dilutions were made in PBS. The number of CFU of *H. ducreyi* present in the suspensions was determined by spreading 50-μl portions of each suspension on chocolate agar plates according to the procedure of Odumeru et al. (48).

Two independent virulence tests were performed, involving a total of 16 rabbits. The *H. ducreyi* cell suspensions in PBS were injected intradermally into the shaved backs of eight rabbits in each experiment, using an injection volume of 0.1 ml. Each rabbit was injected with both the wild-type strain and the mutant strain at different sites. In both experiments, the inoculation sites were observed, palpated, and photographed at 2, 4, and 7 days postinoculation. A numeric value was assigned to the lesions according to the method of Purcell et al. (54). This scoring system assigned the following values to clinical characteristics of the lesions: 0 = no change, 1 = erythema, 2 = induration, 3 = nodule, 4 = necrosis. For each lesion at each time point, the score was given for the most advanced characteristic (i.e., a lesion that was erythematous and indurated was assigned a value of 2 because induration is a more advanced stage). To prevent bias in scoring, strain identities were encoded prior to injection. Mean lesion scores (±standard deviations) for the inoculum sizes of 10^4 and 10^5 CFU were determined on days 2, 4, and 7.

Statistical analyses. Because the lesion scores were not normally distributed, all of the scores for an experiment were ranked from highest to lowest and the analyses were performed on the ranks rather than on the raw data, as recommended (9). These data were analyzed using repeated measures analysis of variance (20), which adjusts for the correlations that arise when the same subject is measured repeatedly with respect to certain factors. In the animal experiments in this study, the repeated factors were the strain used for challenge, the day of observation, and the size of the inoculum.

Recovery of *H. ducreyi* from lesions. After lesions were scored on day 7, each animal was euthanized and then the lesions resulting from the inoculum containing 10^5 CFU were circumferentially excised with a scalpel fitted with a no. 15 blade. Each lesion was dissected, and all the pus was extracted by means of scraping with a sterile toothpick. This material was suspended in 1 ml of sterile PBS, and a small portion (250 μl) of this suspension was used to wash the interior surface of the excavated lesion. After extensive mixing by vortexing, a 0.1-ml portion of this suspension was diluted 1:10 in PBS. Four 50-μl portions of both the original suspension and the dilution were plated on chocolate agar.

Nucleotide sequence accession number. The nucleotide sequence of the *hupA* gene has been deposited at GenBank and was assigned the accession number U34048.

RESULTS

Binding of hemoglobin by *H. ducreyi* strains. Previous experiments involving another heme-requiring pathogen (i.e., *H. influenzae* type b) had revealed that the ability to use a particular source of heme (i.e., heme-hemopexin) was associated with the ability to bind this heme source in a colony blot assay (10, 30). Similarly, recent studies by Lee (40) indicated that *H. ducreyi* could utilize hemoglobin as a source of heme. Testing of a number of wild-type *H. ducreyi* isolates grown on a GC agar-based medium containing heme (hemin) at a final concentration of 12.5 μg/ml revealed that 9 of the 10 strains were able to bind radioiodinated human hemoglobin in a colony blot assay (Fig. 1, columns A to G, I, and K). However, it was also noted that as the concentration of heme in the growth medium was increased, hemoglobin-binding activity was reduced to undetectable levels (Fig. 1), with only one strain (i.e., Cha-1) being able to bind hemoglobin after growth in the presence of heme at 50 μg/ml (Fig. 1, column I). None of these 10 strains could bind hemoglobin after growth in the presence of heme at 100 μg/ml (Fig. 1). *H. ducreyi* strains grown on GC-based agar containing hemoglobin (250 μg/ml) as the sole source of heme also did not exhibit detectable binding of radioiodinated hemoglobin (data not shown).

Of the 10 wild-type *H. ducreyi* isolates tested, only one, 512, exhibited little or no detectable binding of hemoglobin (Fig. 1, column H). It was subsequently found that this strain also was unable to form single colonies when inoculated onto a GC agar medium containing hemoglobin as the sole source of heme. In contrast, this strain readily formed colonies on GC agar medium containing heme in place of hemoglobin (data not shown).

Isolation of a mutant unable to bind hemoglobin. The results of the experiments described above indicated that the well-characterized wild-type *H. ducreyi* strains 35000 and Cha-1 (27, 28, 54) could bind radioiodinated human hemoglobin in a colony blot assay. In an attempt to isolate an isogenic mutant unable to express this hemoglobin-binding activity, both of these strains were mutagenized by using the Tn*1545*-Δ*3*-based transposon mutagenesis system (62). Several kanamycin-resistant strains unable to bind hemoglobin (approximately 1 per 10^3 to 10^4 kanamycin-resistant CFU) were identified in this manner, and SDS-PAGE analysis of cell envelope proteins revealed that these strains all lacked a protein with an apparent molecular mass of approximately 100 kDa that was present in

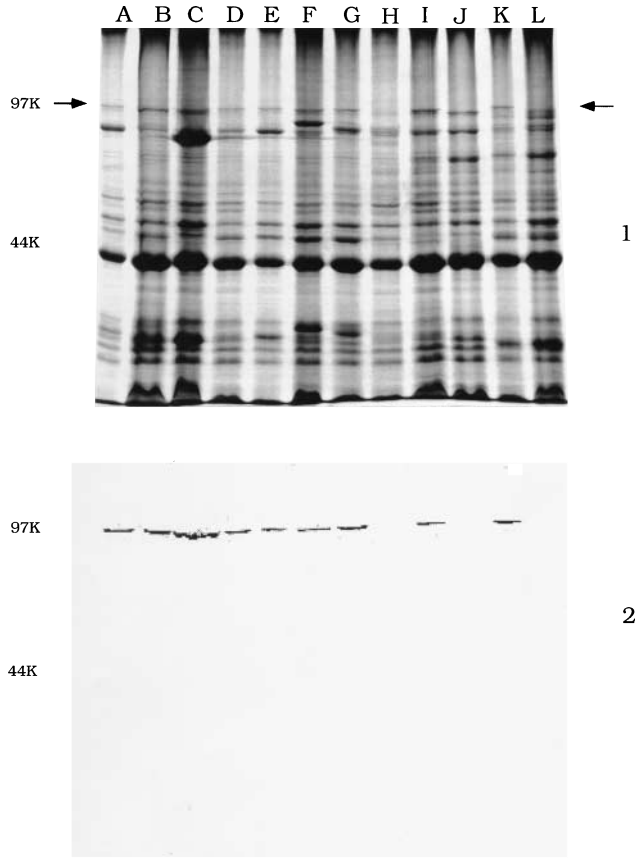

FIG. 2. Cell envelope protein profiles of wild-type and mutant strains of *H. ducreyi*. Proteins present in cell envelopes of the following strains were resolved by SDS-PAGE and either stained with Coomassie blue (panel 1) or transferred to nitrocellulose for Western blot analysis with mouse polyclonal serum raised against the purified 100-kDa protein from *H. ducreyi* 35000 (panel 2). Lanes: A, 1151; B, E1673; C, 041; D, 1145; E, Hd13; F, STD101; G, 1352; H, 512; I, Cha-1; J, Cha-1.1; K, 35000; L, 35000.21. Molecular weight position markers are on the left.

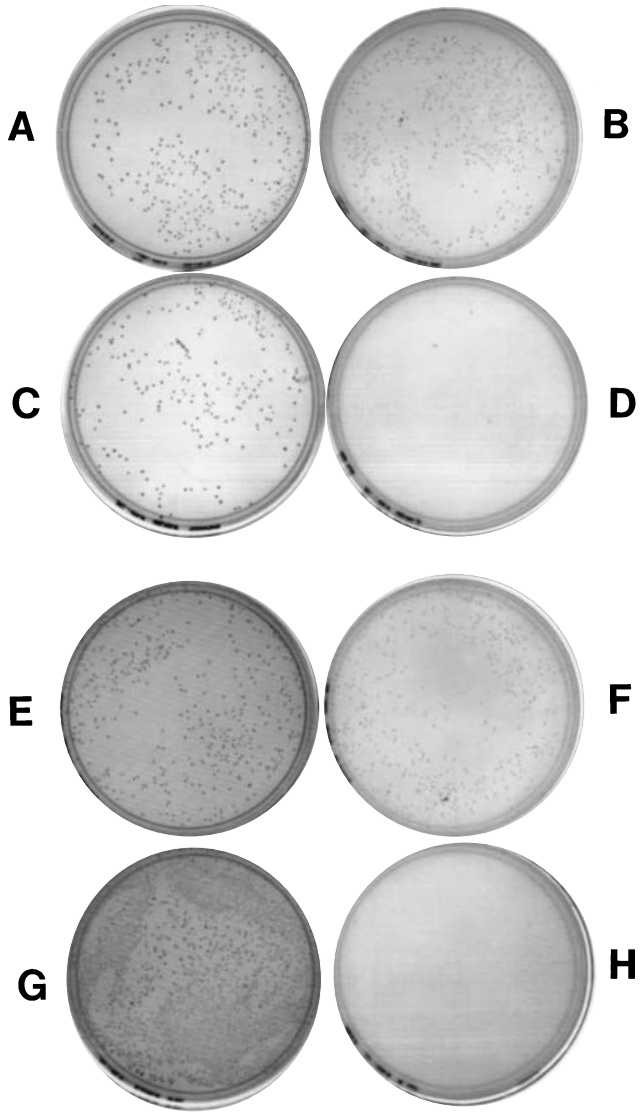

FIG. 3. Comparison of abilities of wild-type and mutant strains of *H. ducreyi* to utilize heme and hemoglobin for aerobic growth. Cells of the wild-type isolates Cha-1 and 35000 and their respective hemoglobin-binding-negative mutants (i.e., the spontaneous Cha-1.1 mutant and the isogenic mutant 35000.21 containing a *cat* cartridge inserted into the *hupA* gene) grown overnight on chocolate agar were suspended in brain heart infusion broth, serially diluted in the same medium, and spread onto GC agar-based plates containing hemin (25 μg/ml) or hemoglobin (250 μg/ml). These plates were photographed after 72 h of incubation. Plates: A, Cha-1 on heme; B, Cha-1 on hemoglobin; C, Cha-1.1 on heme; D, Cha-1.1 on hemoglobin; E, 35000 on heme; F, 35000 on hemoglobin; G, 35000.21 on heme; H, 35000.21 on hemoglobin.

their respective wild-type parent strains. For example, the wild-type Cha-1 strain (Fig. 1, column I) expressed a 100-kDa protein (Fig. 2, panel 1, lane I), whereas the hemoglobin-binding-negative strain Cha-1.1 (Fig. 1, column J) lacked a 100-kDa protein (Fig. 2, panel 1, lane J). Sarkosyl extraction of the cell envelopes of the wild-type parent strains indicated that this 100-kDa protein was apparently localized in the outer membrane (data not shown).

When the proteins present in cell envelopes of the other seven wild-type isolates (i.e., in addition to 35000 and Cha-1) which bound hemoglobin in the colony blot assay were analyzed by SDS-PAGE, it appeared that these seven strains (Fig. 2, panel 1, lanes A to G) all expressed a 100-kDa protein. In contrast, strain 512, which was the only wild-type isolate that did not bind hemoglobin (Fig. 1, column H), apparently lacked a 100-kDa protein (Fig. 2, panel 1, lane H).

When polyclonal mouse antiserum raised against the SDS-PAGE-purified 100-kDa protein from strain 35000 was used to probe whole-cell lysates from the 10 wild-type isolates, antibodies in this serum reacted with a single protein with an apparent molecular mass of approximately 100 kDa expressed by every wild-type isolate (Fig. 2, panel 2, lanes A to G, I, and K) except 512 (Fig. 2, panel 2, lane H). Significantly, this polyclonal antiserum also did not bind any antigens expressed by the Cha-1.1 mutant, which failed to express both hemoglobin-binding activity and the 100-kDa protein (Fig. 2, panel 2, lane J). These data provided presumptive evidence for the involvement of the 100-kDa protein in the binding of hemoglobin by *H. ducreyi.*

Similar to the wild-type isolate (i.e., strain 512), which could not utilize hemoglobin, the hemoglobin-binding-negative strain Cha-1.1 also could not utilize hemoglobin as a source of heme. Whereas the wild-type Cha-1 isolate formed isolated colonies when plated on GC agar-based medium containing either heme (Fig. 3A) or hemoglobin (Fig. 3B), no isolated colonies developed when the hemoglobin-binding-negative

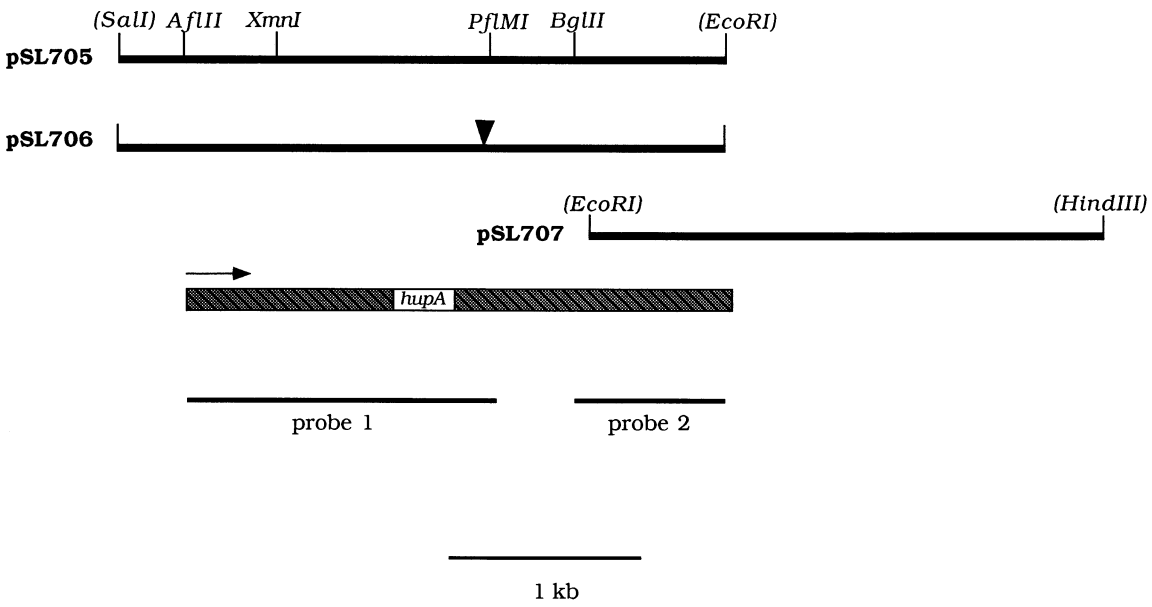

FIG. 4. Partial restriction enzyme map of the *H. ducreyi* chromosomal inserts used for nucleotide sequence analysis and construction of the isogenic *hupA* mutant strain 35000.21. pSL705 is a pBK-CMV-based phagemid with a 3.2-kb insert that contains all of the *hupA* ORF except the last 12 nucleotides. pSL706 is a derivative of pSL705 that has a *cat* cartridge (indicated by the dark triangle) inserted into the *hupA* ORF at the *Pfl*MI site. pSL707 is a pUC19-based plasmid which has a 2.7-kb insert that contains the 3′ end of the *hupA* gene. The extent of the *hupA* ORF is indicated by the cross-hatched bar. Probe 1 demarcates the extent of the 1.6-kb *Afl*II-*Pfl*MI fragment from pSL705 that was used a probe for the *hupA* gene in Southern blot analysis (see Fig. 7 and 9). Probe 2 indicates the 700-bp *Bgl*II-*Eco*RI fragment from pSL705 was used to identify the recombinant bacteriophage from which pSL707 was derived. Restriction sites in parentheses are present in the vector.

strain Cha-1.1 was plated onto GC agar containing hemoglobin (Fig. 3D). In contrast, strain Cha-1.1 readily formed isolated colonies when plated onto heme-containing medium (Fig. 3C).

When a probe for the Tn*1545*-Δ*3* transposon was used for Southern blot analysis of chromosomal DNA from Cha-1.1 and the other kanamycin-resistant, hemoglobin-binding-negative strains, it was found that each strain had the transposon inserted into a different site (data not shown), suggesting that the transposon insertion was not responsible for the observed phenotype in each strain. Additional control experiments, including backcross experiments, proved that the hemoglobin-binding-negative strains derived from both Cha-1 and 35000 were likely spontaneous mutants or variants unable to bind this heme compound. At this point, it was decided that cloning and mutagenesis of the gene encoding the 100-kDa protein was the most direct mechanism for proving the involvement of this protein in the utilization of hemoglobin by *H. ducreyi.*

Cloning of the *H. ducreyi* gene encoding the 100-kDa protein. The SDS-PAGE-purified 100-kDa protein from the wild-type strain 35000 was used to immunize mice whose spleen cells were used in a hybridoma fusion system to produce the 100-kDa-protein-specific MAb 5A9. This MAb was shown to be reactive with the 100-kDa protein expressed by the nine wild-type *H. ducreyi* isolates (Fig. 1) which bound hemoglobin (data not shown). When MAb 5A9 was used to screen a bacteriophage-based genomic library of *H. ducreyi* 35000 DNA, a recombinant clone was identified that bound this MAb. The pBK-CMV-based phagemid that was excised from this recombinant phage contained a 3.2-kb insert of *H. ducreyi* chromosomal DNA and was designated pSL705 (Fig. 4). Western blot analysis of a whole-cell lysate of this recombinant clone showed that it expressed an antigen with an apparent molecular mass of 100 kDa that was reactive with the polyclonal antiserum to the 100-kDa protein (data not shown), a finding which suggested that this clone contained most or all of the structural gene for the 100-kDa protein.

Nucleotide sequence analysis of the gene encoding the 100-kDa protein. The 3.2-kb *H. ducreyi* insert in the recombinant clone pSL705 was shown to contain a large (nucleotides 309 to 3215 in Fig. 5) open reading frame (ORF) that encoded a protein with a calculated molecular weight of 110,595. However, this ORF lacked a termination codon at its 3′ end, a finding which indicated that the insert in pSL705 lacked some small part of the relevant ORF. To obtain the end of this ORF, a 700-bp *Bgl*II-*Eco*RI fragment comprising the 3′ end of the ORF was excised from pSL705 and used to screen a genomic library of *H. ducreyi* DNA constructed in the plasmid vector pUC19. A recombinant plasmid containing a 2.7-kb insert bound this probe and was designated pSL707 (Fig. 4). Nucleotide sequence analysis of the insert in pSL707 provided the 3′ end of the relevant ORF (nucleotides 3216 to 3227 in Fig. 5) and revealed that the insert in pSL705 lacked only 12 nucleotides necessary to complete the ORF. Two inverted nucleotide repeats that resembled rho-independent terminator sequences were located 3′ from the stop codon (Fig. 5). The protein encoded by this ORF was designated hemoglobin utilization protein A (HupA).

The complete *hupA* ORF encoded a predicted protein with a calculated molecular weight of 110,954 that began with a hydrophobic amino acid sequence similar to that of leader peptides (60, 68). A putative signal peptidase I cleavage site (i.e., Val-Tyr-Ala) was located at amino acids 20 to 22 (Fig. 5), such that the predicted molecular weight of the mature HupA protein was 108,590. N-terminal amino acid analysis of SDS-PAGE-purified HupA protein from strain 35000 confirmed the existence of the proposed leader peptide (Fig. 5). The carboxy-terminal residue of the HupA polypeptide was phenylalanine, a finding consistent with the presence of HupA in the *H. ducreyi* outer membrane (67).

The HupA protein has homology to TonB-dependent outer membrane proteins. When the amino acid sequence of the mature HupA protein was compared with those of other pro-

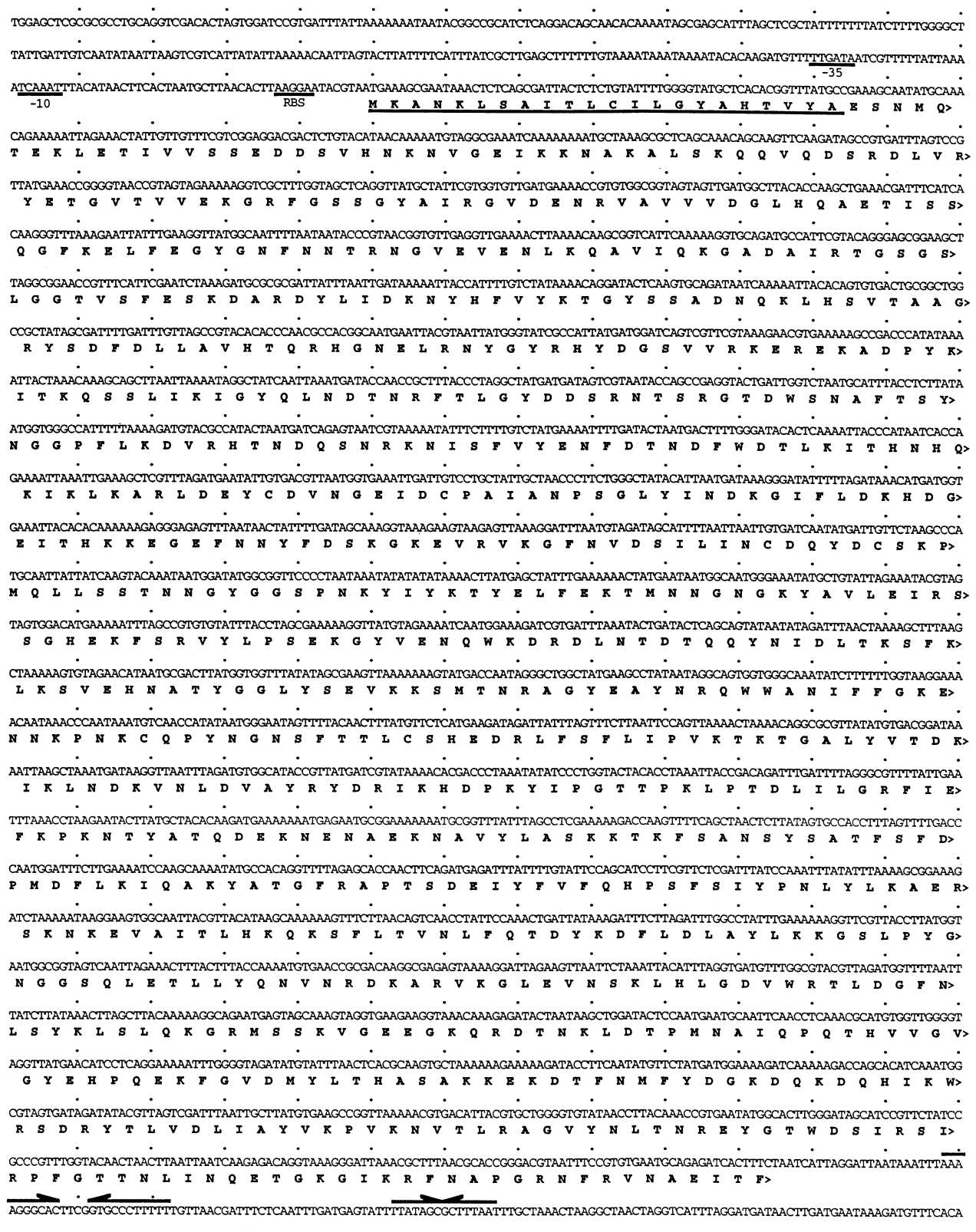

FIG. 5. Nucleotide sequence of the gene encoding the HupA protein of *H. ducreyi* 35000, together with the deduced amino acid sequence of the HupA protein. Putative −35 and −10 regions are underlined, as is the putative ribosomal binding site (RBS). The leader peptide of the HupA protein is underlined. Inverted nucleotide repeats at the end of the *hupA* gene are indicated. The nucleotide sequence of the *hupA* ORF in pSL705 was terminated at nucleotide 3215 by the vector cloning site.

			% Similarity	% Identity
H. ducreyi	HupA	E T I V V S S E D D S V H N		
N. meningitidis	HmbR	E T T P V K A E V K A V R V	55.3	31.2
V. cholerae	HutA	D E V V V S T T R L N T Q I	54.5	30.9
H. influenzae	HxuC	D S I N V I A T R D P S R F	52.1	29.2
N. gonorrhoeae	Tbp1	D T I Q V K A K K Q K T R R	48.2	25.5
Y. entercolitica	HemR	D T M V V T A T G N E R S S	48.0	25.8
N. meningitidis	Tbp1	D T I Q V K A K K Q K T R R	47.8	25.4
P. aeruginosa	PfeA	E Q T V V A T A Q E E T K Q	46.4	23.5
E. coli	FepA	D T I V V T A A E Q N L Q A	45.8	22.6

FIG. 6. Peptide alignment between the TonB box of HupA and the TonB boxes from TonB-dependent outer membrane proteins of other organisms together with the overall similarity and identity between HupA and these other proteins. Amino acid residues in these other TonB-dependent proteins that are identical to those in the HupA TonB box are indicated by shading. The other TonB-dependent proteins included in this analysis are HmbR from *N. meningitidis* (65), HutA from *V. cholerae* (34), HxuC from *H. influenzae* type b (11), Tbp1 from *N. gonorrhoeae* (12), HemR from *Y. enterocolitica* (63), Tbp1 from *N. meningitidis* (41), PfeA from *Pseudomonas aeruginosa* (13), and FepA from *E. coli* (43).

teins in the available data bases, HupA proved to be most similar to TonB-dependent outer membrane proteins, which are usually involved in binding and transport of certain ligands, including heme and iron (32, 53). HupA has a motif near its N terminus that resembled the classic TonB box (31, 43); comparison of the putative HupA TonB box with those of other TonB-dependent proteins is depicted in Fig. 6.

Construction of an isogenic *hupA* mutant. To confirm the involvement of the HupA protein in the binding and utilization of hemoglobin by *H. ducreyi*, an isogenic mutant was constructed that could not express this macromolecule. A *cat* cartridge was ligated into the *Pfl*MI site within the *hupA* structural gene in pSL705, yielding the mutated plasmid pSL706 (Fig. 4). This construct was linearized by digestion with *Pst*I and used to electroporate the wild-type strain 35000. All 52 chloramphenicol-resistant transformants obtained from this procedure were unable to bind hemoglobin in the colony blot assay. One of these chloramphenicol-resistant strains, designated 35000.21 (Fig. 1, column L, and Table 1), was selected for further testing and shown not to express a 100-kDa protein as detected by SDS-PAGE and Coomassie blue staining of cell envelope proteins (Fig. 2, panel 1, lane L). Western blot analysis with the polyclonal 100-kDa-protein (HupA) antiserum confirmed that strain 35000.21 did not express any detectable HupA protein (Fig. 2, panel 2, lane L) whereas the wild-type parent strain expressed readily detectable levels of this protein (Fig. 2, panel 2, lane K).

Southern blot analysis was used to confirm the occurrence of proper allelic exchange in the chloramphenicol-resistant strain 35000.21. When chromosomal DNA from the wild-type parent strain was probed with a 1.6-kb *Afl*II-*Pfl*MI internal fragment from the *hupA* ORF (Fig. 4), an 8-kb *Afl*III fragment hybridized with this probe (Fig. 7, lane A). In contrast, the same *hupA* probe bound to a 9.4-kb *Afl*III fragment from the chloramphenicol-resistant strain 35000.21 (Fig. 7, lane B), a result consistent with the replacement of the wild-type *hupA* gene with the mutated allele containing the 1.4-kb *cat* cartridge. Similarly, the wild-type chromosomal DNA (Fig. 7, lane C) did not bind a *cat* gene probe, whereas a 9.4-kb *Afl*III fragment from strain 35000.21 (Fig. 7, lane D) hybridized with this probe.

Characterization of the isogenic *hupA* mutant. The *hupA* mutant strain 35000.21 was also unable to form single colonies when grown on GC agar containing hemoglobin as the sole source of heme (Fig. 3H). In contrast, this strain readily formed single colonies when heme was provided (Fig. 3G). As expected, the wild-type parent strain could form single colonies on medium containing either heme (Fig. 3E) or hemoglobin (Fig. 3F).

Both the wild-type strain 35000 and the mutant strain 35000.21 were also evaluated for their abilities to bind and utilize hemoglobin-haptoglobin complexes. The wild-type strain bound radioiodinated hemoglobin-haptoglobin in a colony blot assay and also formed isolated colonies when hemoglobin-haptoglobin (16 μg/ml) was provided as the sole source of heme in a GC agar-based medium. In contrast, the mutant strain 35000.21 did not bind radiolabeled hemoglobin-haptoglobin at detectable levels and was unable to form isolated colonies when supplied with hemoglobin-haptoglobin as the sole source of heme (data not shown).

Effect of heme levels on expression of the HupA protein. The hemoglobin-binding assays depicted in Fig. 1 suggested that increasing the level of heme in the growth medium resulted in decreased expression of hemoglobin-binding activity by the various *H. ducreyi* isolates. To determine whether expression of the HupA protein was similarly affected by heme levels, the wild-type strains 35000 and Cha-1 were grown on media containing heme at 12.5, 50, and 100 μg/ml. Western blot analysis of lysates of these cells was performed with both the HupA-specific MAb 5A9 and an MAb (3F12) specific for the major outer membrane protein (61). The latter MAb was included to

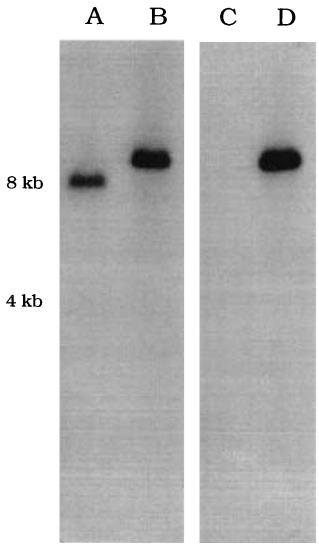

FIG. 7. Southern blot analysis of the wild-type strain 35000 and its isogenic *hupA* mutant strain 35000.21. Chromosomal DNA from strains 35000 (lanes A and C) and 35000.21 (lanes B and D) were digested with *Afl*III, resolved by agarose gel electrophoresis, and probed with a 1.6-kb *Afl*II-*Pfl*MI fragment from pSL705 comprising over half of the *hupA* ORF (lanes A and B) or with the 1.4-kb *cat* cartridge (lanes C and D). Size markers (in kilobases) are on the left.

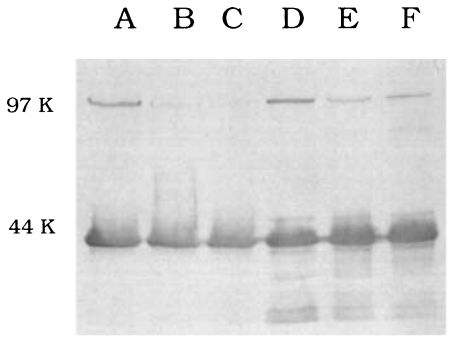

FIG. 8. Effect of heme concentrations in the growth medium on expression of the HupA protein by wild-type isolates of *H. ducreyi*. Strains 35000 (lanes A to C) and Cha-1 (lanes D to F) were grown on GC agar-based media containing heme at final concentrations of 12.5 (lanes A and D), 50 (lanes B and E), and 100 (lanes C and F) μg/ml. Proteins present in whole-cell lysates prepared from these cells were resolved by SDS-PAGE, transferred to nitrocellulose, and probed in Western blot analysis with both the HupA-specific MAb 5A9 and the major outer membrane protein-specific MAb 3F12. Molecular weight position markers (in thousands) are on the left. The major outer membrane protein in this figure is located just beneath the 44-kDa marker, and the HupA protein is located just above the 97-kDa marker.

provide an internal standard for determining the amount of *H. ducreyi* cell material loaded into the Western blot system.

Increasing heme levels in the growth medium virtually eliminated detectable expression of the HupA protein by strain 35000 (Fig. 8, lanes A to C). In contrast, strain Cha-1 still expressed detectable HupA protein even when grown on medium containing heme at 100 μg/ml (Fig. 8, lanes D to F). The latter result correlated with the hemoglobin-binding activity exhibited by strain Cha-1, which was the only wild-type strain which had detectable binding of radioiodinated hemoglobin after being grown on medium containing heme at 50 μg/ml (Fig. 1, column I).

Detection of the *hupA* gene in other *H. ducreyi* strains. Chromosomal DNA preparations from the 10 wild-type isolates and the spontaneous hemoglobin-binding-negative strain Cha-1.1 were digested with *Afl*III and probed with the 1.6-kb *Afl*II-*Pfl*MI fragment from the *hupA* ORF (Fig. 9). Nine of the ten wild-type isolates exhibited a single 8-kb band which bound this probe (Fig. 9, lanes A to I), whereas the HupA-negative strain 512 (Fig. 9, lane J) had a 9-kb fragment reactive with the same probe. In contrast, the spontaneous hemoglobin-binding-negative strain Cha-1.1 (Fig. 9, lane K), which also failed to express the HupA protein, had an 8-kb fragment which hybridized with the *hupA* gene probe and was indistinguishable in Southern blot analysis from that of its wild-type parent strain (Fig. 9, lane H).

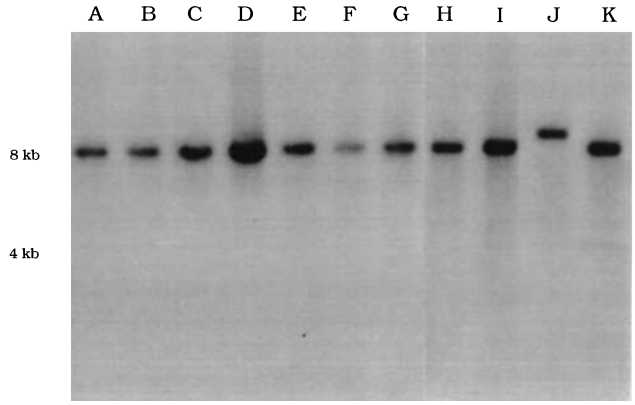

FIG. 9. Detection of the *hupA* gene in wild-type and mutant *H. ducreyi* strains by Southern blot analysis. Chromosomal DNA preparations from the following strains were digested with *Afl*III, resolved by agarose gel electrophoresis, transferred to nitrocellulose, and probed with the 1.6-kb *Afl*II-*Pfl*MI fragment from the *hupA* gene. Lanes: A, 35000; B, 1151; C, E1673; D, 041; E, 1145; F, Hd13; G, STD101; H, Cha-1; I, 1352; J, 512; K, Cha-1.1. Size markers (in kilobases) are on the left.

Virulence of the isogenic *hupA* mutant. To determine whether expression of the HupA protein was involved in virulence, the wild-type strain 35000 and the isogenic *hupA* mutant strain 35000.21 were compared for their relative abilities to produce dermal lesions in the temperature-dependent rabbit model (3, 54). Though not avirulent, the mutant strain proved markedly less able than the wild type to produce lesions in this model system (Table 2). Additional control experiments were performed in which the wild-type 35000 strain was compared with a strain (35000-102) bearing a *cat* cartridge within a gene encoding an irrelevant cell envelope antigen (27). Statistical analysis showed no difference ($P = 0.149$) between the lesion scores obtained with strain 35000-102 and the wild-type 35000 strain (data not shown). It can be inferred from this result that simply the presence of the *cat* cartridge by itself did not affect the virulence potential of *H. ducreyi.*

The importance of the HupA protein to *H. ducreyi* in vivo was reinforced by the results of efforts to recover viable *H. ducreyi* from these skin lesions. When pus was quantitatively recovered from lesions that resulted from injection of inocula containing 10^5 CFU, no viable *H. ducreyi* organisms were obtained from the 16 lesions (8 per experiment) caused by the isogenic *hupA* mutant. In contrast, 10 of the 16 lesions produced by the wild-type strain yielded viable *H. ducreyi*.

In vitro growth rates of the wild-type and *hupA* mutant. To determine whether the difference in virulence between the wild-type strain and the *hupA* mutant might be the result of a limitation in the basic growth rate of the mutant, both strains were inoculated into liquid medium containing heme at a final concentration of 50 μg/ml. The growth rates of the wild-type and mutant strains were indistinguishable under these conditions (Fig. 10). However, when the free heme concentration was reduced to 5 μg/ml, the mutant was unable to grow (Fig. 10).

DISCUSSION

The survival of *H. ducreyi* in vivo is likely related not only to its virulence factors, which potentially include a cytotoxin (55), hemolysin (49), and lipooligosaccharide (8), but also to its ability to acquire those nutrients which allow it to grow in vivo. The fastidious nature of this pathogen has complicated efforts to elucidate its metabolic requirements (1, 46), but the necessity of supplying relatively large amounts of heme or hemoglobin in vitro to allow aerobic growth of this organism is an indication that acquisition of protoporphyrin-iron complexes in vivo may be of paramount importance.

It has been shown that both *H. ducreyi* (40) and the pathogenic *Neisseria* species (15) can utilize free heme, hemoglobin, and hemoglobin-haptoglobin to satisfy their heme requirement in vitro. Interestingly, neither *H. ducreyi* nor these pathogenic neisseriae can utilize heme bound to the serum carrier protein hemopexin (15, 40), whereas most strains of *H. influenzae*, in addition to being able to utilize the aforementioned sources of heme (66), can readily utilize heme-hemopexin complexes as the sole source of heme for growth in vitro (10). Our laboratory has confirmed that *H. ducreyi* cannot utilize heme-hemopexin as a source of heme (data not shown), leaving unan-

TABLE 2. Lesion production by the wild-type 35000 strain and isogenic *hupA* mutant strain 35000.21 in the temperature-dependent rabbit model in two independent experiments[a]

Bacterial strain	Inoculum size (CFU)	Mean lesion score[b]			*P* value[c]
		Day 2	Day 4	Day 7	
Wild-type 35000	10^5	4.00	4.00	4.00	
35000.21 (*hupA* mutant)	10^5	3.88 (±0.33)	3.75 (±0.43)	3.88 (±0.33)	0.0001
Wild-type 35000	10^4	4.00	4.00	4.00	
35000.21 (*hupA* mutant)	10^4	3.00 (±0.50)	2.38 (±0.48)	2.13 (±0.60)	
Wild-type 35000	10^5	4.00	4.00	4.00	
35000.21 (*hupA* mutant)	10^5	4.00	3.75 (±0.43)	3.75	0.0012
Wild-type 35000	10^4	3.63 (±0.70)	3.88 (±0.33)	3.75 (±0.43)	
35000.21 (*hupA* mutant)	10^4	2.75 (±0.83)	1.75 (±0.43)	1.75 (±0.43)	

[a] Eight rabbits were used in each experiment and were infected with both strains simultaneously.
[b] Mean lesion score (±standard deviation).
[c] *P* value calculated for difference between wild-type and mutant lesion scores at both inoculum sizes and on all 3 days of lesion measurement as described in Materials and Methods.

swered the question concerning the molecular basis for this apparent selectivity in heme uptake systems.

The results obtained in this study indicate that *H. ducreyi* has a specialized system for the utilization of heme bound to hemoglobin. Nine of the ten wild-type isolates tested in this study bound hemoglobin in a colony blot assay and could also utilize this protein as a source of heme for growth in vitro. Only one of these isolates (i.e., strain 512) lacked the ability to bind hemoglobin (Fig. 1, column H), and this isolate also failed to utilize hemoglobin as a source of heme for growth. When spontaneous hemoglobin-binding-negative mutants or variants of strains Cha-1 and 35000 were isolated, they were found to lack expression of an outer membrane protein with an apparent molecular mass of 100 kDa (i.e., the HupA protein) (Fig. 2, lane J) and also could not utilize hemoglobin for growth (Fig. 3D). Attempts to demonstrate direct binding of radiolabeled hemoglobin by the HupA protein transferred to nitrocellulose after SDS-PAGE were unsuccessful (data not shown).

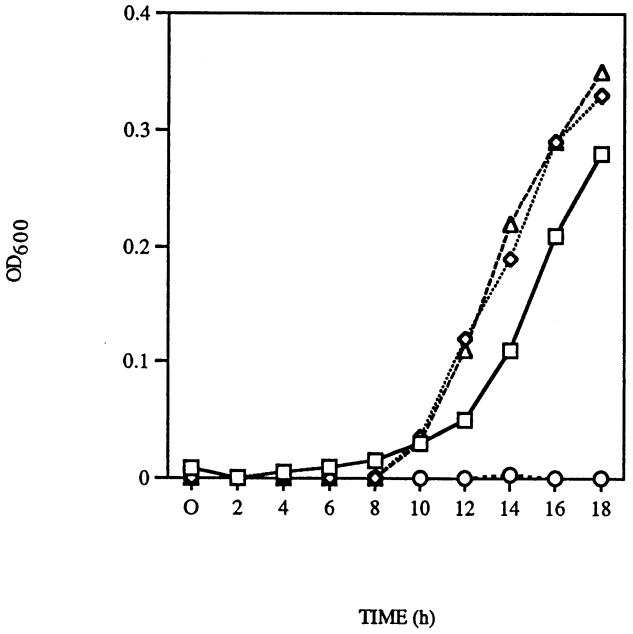

FIG. 10. Growth of the wild-type and *hupA* mutant strains in broth medium containing different levels of free heme. Heme-starved cells of these two strains were inoculated into broth medium as follows: ◇, wild-type 35000, 50 μg of heme per ml; □, wild-type 35000, 5 μg of heme per ml; △, *hupA* mutant, 50 μg of heme per ml; ○, *hupA* mutant, 5 μg of heme per ml.

Cloning and nucleotide sequence analysis of the relevant *H. ducreyi* gene revealed that the 108-kDa HupA protein was similar to TonB-dependent outer membrane proteins (Fig. 6), resembling most closely the HmbR protein of *Neisseria meningitidis*, which is essential for the utilization of hemoglobin-bound iron by this pathogen (65). TonB-dependent outer membrane proteins have been shown to be involved in the uptake of heme and heme compounds by several other pathogens, including *Yersinia enterocolitica* (63, 64), *Vibrio cholerae* (33, 34), and *H. influenzae* (11). With both *Y. enterocolitica* (38, 63) and *H. influenzae* (36), a functional TonB gene product has been shown to be essential for the utilization of heme.

Mutant analysis was used to confirm that the HupA protein played an essential role in the utilization of hemoglobin by *H. ducreyi*. Insertional inactivation of the *hupA* gene resulted in loss of expression of this outer membrane protein (Fig. 2, lane L), an inability to bind radiolabeled hemoglobin (Fig. 1, column L), and a failure to utilize hemoglobin as the sole source of heme for growth (Fig. 3H). Equally important is the fact that this isogenic *hupA* mutant could not utilize hemoglobin-haptoglobin in vitro. While free hemoglobin can be utilized readily by *H. ducreyi* in vitro, the small amount of circulating hemoglobin in vivo (i.e., that not present in erythrocytes) is tightly complexed (K_d, $\sim 10^{-23}$ M) by the serum protein haptoglobin (6). The heme source(s) actually utilized by *H. ducreyi* growing in vivo is open to speculation, but the production of a hemolysin by this pathogen (49) suggests that *H. ducreyi* is equipped to disrupt erythrocytes, thereby releasing hemoglobin which, even after its binding by haptoglobin, can be acquired by wild-type *H. ducreyi*. The fact that the *hupA* mutant cannot utilize hemoglobin bound to haptoglobin reinforces the importance of the HupA protein to the acquisition of heme by this pathogen.

The only previous report of a hemoglobin-haptoglobin-binding outer membrane protein is of that from *N. meningitidis*, as described by Dyer and coworkers (42). A mutant lacking expression of this 85-kDa hemoglobin-haptoglobin-binding protein was unable to utilize hemoglobin-haptoglobin as a source of iron for growth in vitro and was also impaired in its ability to utilize hemoglobin (42).

It is noteworthy that a mutation inactivating expression of the *hupA* gene resulted in a statistically significant diminution of the virulence of *H. ducreyi*, as assessed in an animal model for dermal lesion formation (Table 2). While other relatively avirulent strains of *H. ducreyi* have been described (3, 25), to the best of our knowledge, this is the first report of a specific mutation which attenuates the virulence of *H. ducreyi*. The fact that the isogenic *hupA* mutant was less able than the wild-type strain to produce dermal lesions and survive in vivo underscores the importance of heme acquisition to this pathogen. At the same time, the fact that the *hupA* mutant was not avirulent in this animal model indicates that *H. ducreyi* likely possesses alternative means for obtaining protoporphyrin-iron complexes when growing in vivo.

The ability of the isogenic *H. ducreyi hupA* mutant to utilize free heme (Fig. 3) indicates that this mutant still possesses a mechanism for the uptake and utilization of this protoporphyrin-iron complex when it is not present in hemoglobin. The molecular nature of this uptake system is not known, although it is tempting to speculate that there may exist another outer membrane protein responsible for utilizing free heme and perhaps heme extracted from carrier proteins such as hemoglobin. In this regard, it should be noted that *H. influenzae* expresses at least three different systems for obtaining heme (10, 11, 21).

While *H. ducreyi* likely contains a receptor(s) for free heme, it is somewhat puzzling that the *hupA* mutant was unable to grow on low levels of free heme in broth media (Fig. 10). The fact that the wild-type and *hupA* mutant strains grew at the same rate in broth containing free heme at 50 μg/ml indicates that there is no detectable difference in the basic growth rates of these two strains in vitro. However, when the concentration of free heme was reduced by an order of magnitude, only the mutant strain was unable to grow (Fig. 10). This last result raises at least two interesting possibilities. The first is that the HupA protein is actually a heme receptor and binds hemoglobin via the heme moiety. This possibility seems unlikely because it has been shown that free heme does not significantly inhibit binding of hemoglobin to *H. ducreyi* (17). Alternatively, it is possible that the true heme receptor in the *H. ducreyi* outer membrane is located immediately adjacent to the HupA protein and perhaps even exists in a complex with HupA. In the latter scenario, lack of HupA expression would affect the orientation of the heme receptor and compromise its functional ability. A somewhat analogous situation has been described for *Neisseria gonorrhoeae*, in which independent mutations in the *frpB*, *tbp*, and *lbp* genes adversely affected heme-bound iron utilization by the gonococcus (5). Nonetheless, the fact that lack of HupA expression resulted in both an inability to utilize hemoglobin and a reduced ability to utilize low levels of free heme reinforces the importance of HupA to heme acquisition. Moreover, this further diminution of heme uptake ability may have contributed to the reduced ability of the *hupA* mutant to produce dermal lesions in animals.

It is also of interest that the level of heme in the growth medium affected expression of the HupA protein differently in two *H. ducreyi* strains. Increasing the concentration of heme from 12.5 to 50 μg/ml virtually eliminated detectable expression of HupA by strain 35000 (Fig. 8, compare lanes A and B), whereas strain Cha-1 still expressed detectable levels of HupA even when grown in the presence of heme at 100 μg/ml (Fig. 8, lane F). These data correlated with results of the colony blot assays, in which strain Cha-1 (Fig. 1, column I) was the only strain among those tested that still exhibited detectable binding of radiolabeled hemoglobin after growth in the presence of heme at 50 μg/ml. The regulatory factor(s) involved therein remains to be identified but may involve heme or the iron derived from heme or both.

The molecular basis for the lack of expression of the HupA protein by both the wild-type isolate 512 and the spontaneous hemoglobin-binding-negative strains is not known. The isolation of what appeared to be spontaneous *hupA* mutants from two different wild-type strains (i.e., Cha-1 and 35000) indicates that expression of HupA may not be stable in vitro. In addition, the frequency of isolation of these spontaneous hemoglobin-binding-negative strains (ca. 1 per 10^3 to 10^4 CFU) appears to be rather high for normal spontaneous mutation. Whether there may exist some type of phase variation in expression of HupA remains to be determined.

The isolation of these spontaneous hemoglobin-binding-negative strains from two different HupA-expressing wild-type strains does raise the possibility that the wild-type strain 512, which does not express HupA, is actually a spontaneous mutant or some type of phase variant. It should also be noted, however, that strain 512 was the only wild-type isolate that exhibited some restriction fragment length polymorphism with respect to the *hupA* gene (Fig. 9, compare lane J with the other lanes). It is possible, therefore, that strain 512 has a more significant mutation (e.g., an insertion or deletion) that is responsible for its lack of expression of HupA.

As this study was being completed, two papers were published by Elkins and colleagues (17, 18) in which they identified a high-molecular-weight hemoglobin-binding protein, designated HgbA, which was required for hemoglobin utilization by *H. ducreyi*. Comparison of the nucleotide sequence of the *hgbA* gene with that of the *hupA* gene revealed only five nucleotides that differed, and these differences resulted in only two amino acid changes, indicating that the HgbA and HupA proteins are essentially identical. In contrast to the results of the present study, these other workers detected the HgbA/HupA protein in all strains of *H. ducreyi* tested.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant AI32011 to E.J.H. and J.D.R. M.K.S. was the recipient of a National Research Service Award (F32-AI08848).

We thank Jo Latimer for superb technical assistance. We also thank Joel Bazue, Robert Munson, and Christopher Elkins for helpful discussions.

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Editor: B. I. Eisenstein